Microaerophilic production of alginate by *Azotobacter vinelandii*

Mikroaerophile Alginatproduktion mit *Azotobacter vinelandii*

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Wael Sabra
aus Alexandria, Ägypten

1. Referent:	Prof. Dr. W.-D.Deckwer
2. Referent:	Prof. Dr. Ing. D. C. Hempel
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LIST OF CONTENT

SYMBOLS AND ABBREVIATIONS

A.	Azotobacter	
A'_{mix}	the absorbence of 50µg alginate sample without borate at 55°C	
A_1 A_2	the absorbence of 50µg of mannuronic acid or guluronic acid without borate at 55°C	
A_1' A_2'	the absorbance of 50µg of mannuronic acid or guluronic acid with borate at 55°C	
ADP	adenosine diphosphate	
A_{mix}	the absorbence of 50µg alginate sample with borate at 55°C	
ATP	adenosine triphosphate	
b		
C.M.	complex, not defined medium	
C4C5		
C_{alg}	alginate concentration	($g\ l^{-1}$)
CoA	coenzyme A	
C_{PHB}	PHB concentration	($g\ l^{-1}$)
C_{rec}	carbon recovery	($g\ g^{-1}$)
D	dilution rate	(h^{-1})
DSM	Deutsche Sammlung von Mikroorganismen	
DW	dry weight	
Eq.	Equation	
F_1 or $_2$	the fraction of the first or second uronic acid in the alginate sample	(%)
FADH2	reduced flavin adenine dinucleotide	
Fig.	figure	
F_m	medium flow rate	($l\ h^{-1}$)
G	guluronic acid residue	
g	gram	
h	hour	
K	consistency index	($mPas^n$)
l	liter	
M	mannuronic acid residue	
m_X^{t2}	biomass concentration at t_2	
m_X^{t1}	biomass concentration at t_1	
m_{alg}^{t2}	alginate concentration at t_2	
m_{alg}^{t1}	alginate concentration at t_1	
m_{PHB}^{t2}	PHB concentration at t_2	
m_{PHB}^{t1}	PHB concentration at t_1	
m_s	substrate used for maintenance	($g\ g^{-1}\ h^{-1}$)
m_o	oxygen consumption for maintenance	($mmol\ g^{-1}\ h^{-1}$ or $g\ g^{-1}\ h^{-1}$)
n	flow behaviour index	
N.F.M	nitrogen free mediums	
NAD+	nicotinoamide adenine dinucleotide	
NADH2	reduced nicotinoamide adenine dinucleotide	
OD	optical density	
OUR	oxygen uptake rate	
P	product (Alginate)	

PHB	poly -ß- hydroxybutrate	
PO_2	dissolved oxygen concentration	
Q		
q_{alg}	specific alginate formation rate	($g\ g^{-1}\ h^{-1}$)
Q_{alg}	production rate of alginate	($g\ l^{-1}h^{-1}$)
q_{CO2}	specific CO_2 production rate	($mmol\ g^{-1}\ h^{-1}$ or $g\ g^{-1}\ h^{-1}$)
Q_{CO2}	volumetric carbon dioxide production rate	($mmol\ l^{-1}\ h^{-1}$ or $g\ l^{-1}\ h^{-1}$)
q_{O2}	specific oxygen uptake rate	($mmol\ g^{-1}\ h^{-1}$ or $g\ g^{-1}\ h^{-1}$)
Q_{O2}	volumetric oxygen consumption rate	($mmol\ l^{-1}\ h^{-1}$ or $g\ l^{-1}\ h^{-1}$)
q_{PHB}	specific PHB production rate	($g\ g^{-1}\ h^{-1}$)
Q_{PHB}	production rate of poly ß hydroxybutyrate	($g\ l^{-1}h^{-1}$)
q_s	specific consumption rate of sugar	($g\ g^{-1}\ h^{-1}$)
$q_{(sm)}$	specific substrate uptake rate for maintenance	($g\ g^{-1}\ h^{-1}$)
$q_{(as)}$	specific substrate uptake rate for assimilated carbon	($g\ g^{-1}\ h^{-1}$)
Q_S	consumption rate of substrate	($g\ l^{-1}h^{-1}$)
Q_X	production rate of biomass	($g\ l^{-1}h^{-1}$)
R	reactivity ratio	
RPM	revolution per minute	
RQ	respiratory quotient	
S	substrate	
S_F	sugar concentration in the fed medium	($g\ l^{-1}$)
S_R	residual sugar concentration in the culture medium	($g\ l^{-1}$)
Tab.	table	
TCA	tricarboxylic acid cycle	
UBICON	universal bioprocess control system	
V_G	total aeration rate	($l\ h^{-1}$)
V_L	working volume of the bioreactor	(l)
V_N	molar volume of ideal gasses	($l\ mol^{-1}$)
w^C_X	carbon content of biomass	($g\ g^{-1}$)
w^C_{alg}	carbon content of alginate	($g\ g^{-1}$)
w^C_{PHB}	carbon content of PHB	($g\ g^{-1}$)
w^C_{CO2}	carbon content of carbon dioxide	($g\ g^{-1}$)
$w^C_{sucrose}$	carbon content of sucrose	($g\ g^{-1}$)
X	pure biomass concentration without PHB	($g\ l^{-1}$)
X_{total}	biomass dry weight with the intracellular PHB	($g\ l^{-1}$)
X^{in}_{CO2}	molar fraction of CO_2 in inlet gas mixture	($mol\ mol^{-1}$)
X^{out}_{CO2}	molar fraction of CO_2 in outlet gas mixture	($mol\ mol^{-1}$)
X^{in}_{O2}	molar fraction of O_2 in inlet gas mixture	($mol\ mol^{-1}$)
X^{out}_{O2}	molar fraction of O_2 in outlet gas mixture	($mol\ mol^{-1}$)
$Y_{alg/X}$	alginate yield on produced biomass	($g\ g^{-1}$)
$Y_{PHB/X}$	PHB yield on produced biomass	($g\ g^{-1}$)
$Y_{X/O}$	biomass yield on oxygen consumed	($g\ mmol^{-1}$ or $g\ g^{-1}$)
$Y_{X/S}$	biomass yield on consumed sugar	($g\ g^{-1}$)

Greek Symbols

µ	growth rate	(h^{-1})
$µ_{max}$	maximum growth rate	(h^{-1})
α	fraction of sucrose which is carbon	

β	fraction of biomass which is carbon	
ϕ	fraction of alginate which is carbon	
ΔS	total substrate utilised	
$\Delta S_{(biomass)}$	substrate utilised for biomass	
$\Delta S_{(alginate)}$	substrate utilised for alginate	
$\Delta S_{(m)}$	substrate utilised for maintenance	
γ	shear rate	(s^{-1})
τ	shear stress	(pa)

1. INTRODUCTION AND AIM OF WORK

All alginates used for commercial purposes are currently being produced by the harvesting of brown seaweeds. However, considering the quality of bacterial alginate and the environmental impact associated with seaweed harvesting and processing, it is more probable that bacterial alginate may become commercial products. Furthermore, alginate with unique qualitative properties has the advantage that it may potentially be sold at higher prices and this may open new markets for this polymer.

Several bacteria belonging to the genera *Pseudomonas* and *Azotobacter* can synthesise alginate. Considering pathogenicity associated with species of *Pseudomonas* and in view of its potential exploitation as food and pharmaceutical additives, *A. vinelandii* appears to be more suitable for a commercial alginate production.

For an efficient conversion of substrate to alginate by *A. vinelandii*, an accurate control of the dissolved oxygen tension must be established which comprises a challenging problem facing alginate production in bioreactor. Higher oxygen concentrations waste the substrates in respiration (as CO_2) in the so called 'nitrogenase protection' while low concentrations of oxygen activate the formation of poly –ß-hydroxybutyrate (PHB).

Because of the difficulty of controlling the dissolved oxygen tension at lower values in bioreactors (Burke et al. 1998), most basic work dealing with the microbial production of alginate by *Azotobacter vinelandii* had been done under fully uncontrolled conditions (in shake flasks (Jarman et al. 1978; Brivonese and Sutherland 1989; Savalgi and Savalgi 1992; Clemeniti et al. 1995; Pena et al. 1997) or with the help of agitation speed (Parente et al. 1998) and contradictory results were routinely cited in literature. For this purpose the influence of oxygen supply on cell growth, metabolism and alginate formation should be quantitatively studied at first, in order to find out the influence of other nutrient limitations on process optimisation.

A. vinelandii belongs to the rare group of nitrogen fixing organisms which live aerobically. Since the nitrogenase enzyme is irreversibly inactivated by oxygen, the bacterium employs two mechanisms for protecting its nitrogenase. Respiratory protection and conformational protection were reported to be the two protection mechanisms and no correlation between

alginate production as a capsule and nitrogen fixation was reported in the literature. For this purpose a detailed morphological study with quantitative analysis of the microaerobic culture kinetics is required for understanding the interdependency of growth, substrate utilisation, PHB, alginate formation and nitrogen fixation.

Aim of the work.

The general aim of the presented work is to optimise alginate production in bioreactor in terms of quantity and quality. Knowing the biological role of alginate in this bacterium will be of great help for understanding and optimising the process.

In order to realise the aim mentioned above, this thesis has been focused on the following main tasks:

- Identifying the optimal pO_2 spectra for the production of alginate by this strain under fixed agitation speed in bioreactor under defined conditions.

- Analysing the kinetics of alginate production under varied initial concentrations of phosphate in DOT-controlled and uncontrolled system in order to clarify the contradictory opinions in literature and to asses the potential for improving alginate production rates.

- Evaluating the effects of phosphate limitation and different DOT values on alginate production as well as on the molecular weight and polymer chemical structure in chemostat culture. The findings form this continuous culture should explain some of the discrepancies concerning the optimum DOT value for alginate production. Other control parameters, beside the control of DOT, for controlling alginate production by *A. vinelandii* were also desired.

- Growth at carbon and energy source limitation in a fed batch culture and its effect on alginate productivity and rheological properties.

- Understanding and predicting the biological role of alginate for *Azotobacter vinelandii* growing diazotrophically through morphological studies in continuous culture with

different agitation speeds and different DOT values. Finding out whether hydrodynamic effect on alginate production exist at different stirring speed was also desirable.

From the previous tasks, a better understanding of the process optimization should be achieved. Furthermore, the narrow range of DOT reported for the best alginate productivity by this bacterium and the task of product quality manipulation should be clarified.

2. THEORETICAL BASIS AND LITERATURE REVIEW

2.1. Alginate

2.1.1. Bacterial and algal sources

Alginate belongs to a family of unbranched binary copolymers of (1-4) linked ß-D-mannuronic acid (M) and α-L-guluronic acid units (G), the relative amounts of which vary greatly between alginates from different species of algae, or between the different regions in the same alga. Additionally, alginic acid from different sources varies in the arrangement of the uronic acids within the molecule. Except for some bacterial polyuronides, alginates are true block copolymers composed of homopolymeric regions of M and G units, termed M and G blocks interspaced with regions of alternating structures of MG blocks. (**Fig. 2.1**).

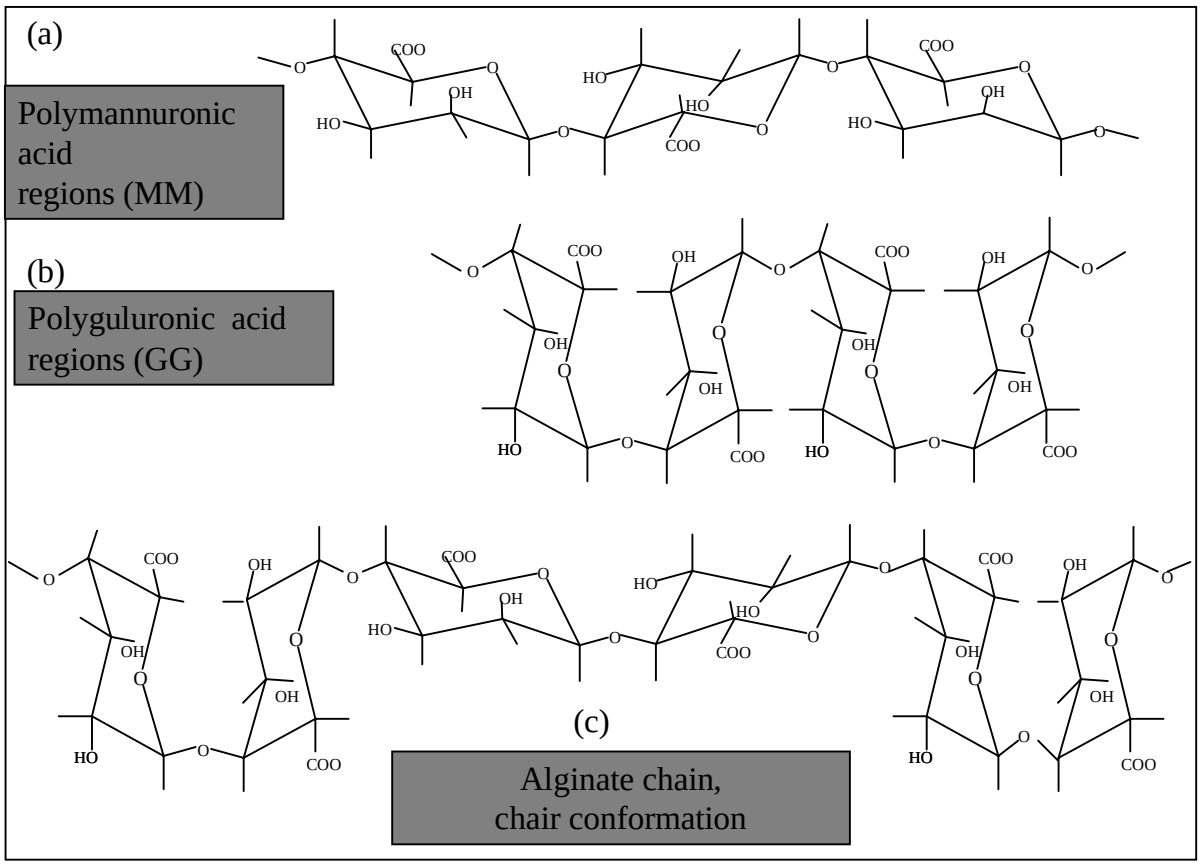


Fig. 2.1 Secondary structure of polymannuronate (a), polyguluronate (b) and the alginate chain, chair conformation (after Moe et al. 1995)

This polyuronide is well known and the present source of commercial alginate is the giant brown kelp *Macrocystis pyrifera.* Because only a few species of brown algae are suitable for

commercial alginate production, in respect to abundance, location and uniform quality, there is a present interest in an alternative bacterial alginate. Microbially derived alginates are under development and their future is very promising.

Species of *Pseudomonas* and *Azotobacter* are the only prokaryotic sources for this algal like-polymer. *Pseudomonas aeruginosa* (a human pathogen causing chronic respiratory infections of cystic fibrosis patients) was first reported to produce this polysaccharide being important for the virulence of this strain and its survival in the lung. Also several species of the genus *Pseudomonas* (*Pseudomonas mendocina* and *Pseudomonas syringae*) have the ability to produce alginate under several conditions. Many strains of *Azotobacter vinelandii* (a nitrogen fixing soil bacterium) were also found to produce this polymer in complex and synthetic media.

The alginates produced by bacteria were, in all cases, reported to be rich in mannuronic acid. Some spp. of pseudomonads have the ability to form a polymannuronate polymer lacking guluronic acid residues totally (Franklin et al. 1994). On the other hand, algal alginate varies in proportion from ca. 25% to75% of D- mannuronic acid.

Unlike algal alginates, bacterial alginates are partly *o*-acetylated, with the acetyl groups being located solely on the mannuronic acid residues. These acetyl groups are suggested to play a role in protecting certain mannuronic acid residues from epimerisation to guluronic acid (Davidson et al. 1977).

2.1.2. Physical properties

2.1.2.1. Ion Binding

The ion binding properties of alginates are the basis for their gelling properties. Alginates show characteristic ion-binding properties in that their affinity for multivalent cations depends on their composition. Characteristic affinities were shown to be a property exclusive to polyguluronate, while polymannuronate was almost without selectivity.

The high selectivity between ions as similar as the alkaline earth metals indicates that the mode of binding could not be by non-specific electrostatic binding only, but that some chelation caused by structural features in the G-blocks must contribute to the selectivity. This characteristic property was explained by the so-called 'egg-box' model based upon the linkage conformations of the guluronate residues. NMR studies suggested a possible binding sites for Ca^{2+} ions in a single alginate chain as shown in **Fig. 2.2** (Moe et al. 1995).

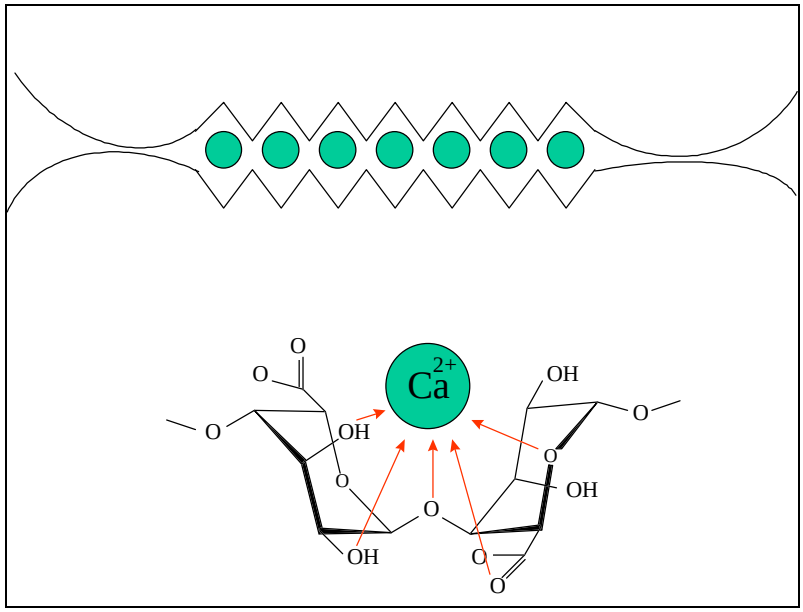


Fig. 2.2 The egg-box model for binding of divalent cations to alginate and the possible chelation of ions by GG sequences. (after Moe at al. 1995)

2.1.2.2. Solubility

The three essential parameters which determine the solubility of alginate in water after Moe et al. (1995) are:

⇒ The pH of the medium. Lowering the pH of the medium leads to a precipitation of the alginate within a relatively narrow pH range depending on the molecular weight of the alginate.

⇒ The ionic strength of the medium. Alginate may be precipitated and fractionated by high concentrations of inorganic salts like potassium chloride. On the other hand, salt concentration of less than 0.1M is sufficient to slow down the kinetics of the dissolution process and hence limits the solubility.

⇒ Effect of gelling ions. At Ca^{2+} concentrations below 3mM, almost all alginate is found within the supernatant, whereas almost no alginate is present in solution when the free Ca^{2+} ion concentration exceeds 3mM.

2.1.2.3. Rheology

Alginate solutions are in general highly viscous. This, however, is caused by the extended conformation of the alginate molecule, giving alginate a large hydrodynamic volume and high ability to form viscous solutions. The intrinsic viscosity of alginates is shown to be dependant on the conformation (their molecular weight, compositions, and sequence of M and G units) and on the ionic strength of the solution (Lebrun et al.1994; Moe et al. 1995).
Alginate solutions in general are pseudoplastic and thus the apparent viscosity is shear rate dependant and can be defined by the power low

$$\tau = K \gamma^n$$

where K is the consistency index (apparent viscosity at a shear rate of 1 s^{-1}), γ is the rate of shear and n is the flow behaviour index. The higher the value of K, the more viscous the fluid. The flow behaviour index varies from 1 to 0 for pseudoplastic fluids; the lower its value the more pronounced is the pseudoplasticity of the fluid.

2.1.2.3.1 Alginate as a gel

In general, the type of alginate gel depends on the number and strength of the cross links and on the length and stiffness of the chains between cross links. The modulus of Ca-alginate gel depends strongly on the composition and sequence of the monomers in the alginate molecule. Alginates rich in guluronate residues form strong, dense gels, while M-rich alginates form softer and elastic gels (Matsumoto et al. 1992). For this reason only alginates having G blocks (algal and *Azotobacter* alginates) can bind calcium to form rigid gels, while *pseudomonads* alginate form only soft gels because of the lack of G blocks in the polymer.

2.1.3. Advantage of producing microbially derived alginate

Current commercial production of alginates uses seaweeds as the polymer source. The polymer has been found in varying amounts in different parts of the alga and the yield depends on the season of the year. The proportion of the two sugar residues varies from one sample to another with resultant differences in the properties of alginates.

Alginate from *Azotobacter vinelandii*, however, can be produced in which the ratio of the uronic acid can be controlled to some extent (Annison and Couperwhite, 1986 a,b). Thus bacterial alginate with appropriate specification should be capable of replacing algal alginate in many of its applications.

Furthermore, polymannuronic acid (100% mannuronan), a substance used as immunostimulant, cannot be prepared from brown algae due to the inherent 'contamination' by guluronic acid. In contrast, the production of alginate by bacteria can more easily be driven to a pure polymannuronic acid by elimination of the epimerase gene (Franklin et al. 1994).

As compared to bacterial alginate, a five fold higher concentration of algal alginate is required to reach the same viscosity at low shear rates (Chen et al. 1985). Also, a solution of bacterial alginate is more pseudoplastic than that of algal alginate. Thus the cost of alginate for various uses could be reduced dramatically if bacterial alginates were chosen. Pseudoelasticity could also provide advantages in processing, such as in pumping and filling. Bacterial alginate could thus result in a pleasing texture in the mouth and excellent flavour release when the product is used in foods. However it is still unknown whether the pseudoelasticity of bacterial alginate is advantageous to industries other than the food industry (Chen et al. 1985).

The production of alginate by microbial fermentation would also have the considerable advantage of assured yield of known composition, being unaffected by marine pollution and tides, and the location of production can also be arranged to utilise convenient or cheap substrates. It is worth noting that the global consumption of alginates in 1985 was 23,000 tons with a marketing value of 115 million $/year (Yalpani and Sandford, 1987, Sutherland,

1996). Thus, market volume of this seaweed polymer should stimulate the development of an alternative microbial fermentation process to reduce the cost of this polysaccharide.

2.1.4. Application of alginate gels in biotechnology

Alginic acid is a commercially important polysaccharide which has many applications in biotechnology and food industry. In recent years, entrapment within spheres of calcium alginate gel has become the most widely used technique for immobilising living cells (bacteria, cyanobacteria, algae, fungi, yeast, plant protoplasts, plant and animal cells). Alginate immobilised cell systems are used as biocatalysts in several industrial processes ranging from ethanol production by yeast cells to the production of monoclonal antibodies from hybridoma cells. Alginate gel also has a potential as implantation material for hormone-producing cells, and encapsulated langerhans islets are currently being evaluated as a bio-artificial endocrine pancreas (Skjak-Braek, 1992; Crescenzi, 1995; Clementi, 1997).

Alginate is active in stimulating immune cells to secrete cytokines, eg. tumor necrosis factor -α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (Otterlei et al. 1991). Surprisingly, the response of the immune system appears to depend upon the sequential structures of alginates, giving the highest response with M-rich polymers, while the G-blocks appear to be non-stimulating. In fact, guluronic acid residues cannot be accepted in therapeutic preparations because it triggers unwanted effects such as antibody generation. (Skjak-Braek, 1992).

Textile and paper industries use alginate along with other materials as ‘sizes’ to improve the surface properties of cloths and paper. This is important prior to printing to enable deposition and adherence of dyes and ink substances (Sutherland and Ellwood, 1979).

Alginates are also used in water treatment processes since they help in increasing the aggregate sizes in the flocculation processes (Rehm and Valla, 1997).

Alginate is used mainly in food industry, which currently consumes about 50% of the alginate produced. It is used, for example, in ice-creams, frozen custards, as well as cream

and cake mixtures. It also found application in beer manufacture to enhance the foam and fruit drinks to assist the suspension of fruit pulp, which makes the product more appealing to the consumer (Neidleman, 1991).

Alginate has also several miscellaneous applications, such as in pharmaceutical preparations (to form stable emulsion), as dental impression material, in coating of tree roots prior to planting to ensure hydrophilic coating for the roots during transport from the nursery to the planting site, and in coating for fresh citrus fruits as an inert pesticide adjuvent.

2.2. Bacterial formation of alginate

2.2.1. *Azotobacter vinelandii* versus *Pseudomonads* spp.

As an alternative source of algal alginate and for the many advantages of bacterial alginates, many trials have been made for the production of alginate by different strains of *Azotobacter vinelandii* and *Pseudomonas* spp.

In *Pseudomonas* (*P. mendocina*, Sengha et al. 1989) the efficiency of conversion of carbon source to alginate was constant within a wide range of DOT (10-112mm Hg), this contrasts greatly with the behaviour of *A. vinelandii* where the conversion efficiency falls dramatically above a DOT of 4mm Hg (Horan et al. 1983). Such a difference is not surprising because of the involvement of respiratory protection in *Azotobacter* species.

The stable production of the polymer is also a critical factor for evaluating the alginate producing bacterium. The mucoid variants of *Pseudomonas mendocina*, however, are unstable despite the production of copious amounts of alginate (Table 2.1). Hacking et al. (1983) attempted to stabilize polysaccharide synthesis using surfactants, a method used in *P. aeruginosa*, but this was also unsuccessful

Although alginate production was reported first for the opportunistic pathogen *Pseudomonas aeruginosa* and then three non-pathogenic species of *Pseudomonas*, including *P. mendocina*, *P. putida* and *P. fluorescens*, and although *Pseudomonas* strains exhibited higher alginate

yields per unit carbon consumed than *A. vinelandii* strains, the soil bacterium *A. vinelandii* appears to be more appropriate for commercial bacterial alginate production in view of its potential exploitation as a food additive and the potential hazards of pathogenisity associated with *Pseudomonas* (Clementi et al. 1995). Moreover, *pseudomonads* alginates lack polyguluronic block structures found in *A. vinelandii,* which affects gelation properties (Fett and Wijey 1995).

Table 2.1 shows a literature review comparing the different strains used and their respective alginate yields.

2.2.2. Life cycle of *Azotobacter vinelandii*

The cellular differentiation cycle leading to encystment and germination in this strain are presented in **Fig. 2.3.**

Azotobacter vegetative cells undergoing division have a typical 'peanut' shape. Some strains are motile by means of peritrichous flagella. Upon induction of encystment, the cells lose motility, become spherical and their walls become thickened over a period of hours and the developing cysts become optically refractile. The cyst consists of a central body which is encased in an inner coat -the intine- and an outer coat- the exine- separated from the intine by the intine space.

Cyst germination is a slow process during which the central body swells and occupies the intine volume, the growth of the cyst within the exine causes a ring type fracture of the outer cyst coat, then the dividing cell emerges leaving the non utilised exine components as an empty cup like structure (Sadoff, 1975).

Tab. 2.1 Literature review and comparative study of alginate yields of different microbial sources.

Bacterial strain	Sugar used g/l	Alginate g/l	Fermentation mode	Medium	Reference
Azotobacter vinelandii					
Strain AX	40g glucose	6,7	Flasks	C.M.	Brivonese & Sutherland, 1989
Mutant strain C-14	20g sucrose	6,2	Flasks	N.F.M	Chen et al.1985
Strain L1	20g sucrose	6,2	Flask	C.M.	Savalgi & Savalgi, 1992
DSM 576	25g glucose	6,3	Flasks	C.M.	Clementi et al. 1995
Strain SM52B NCIB 9086	20g sucrose	5,6	Flasks	N:F:M	Horan et al.1981
ATTC-9046	20g sucrose	4.5	Flasks	C.M	Pena et al. (1997)
Strain NCIB 9086	40g sucrose	3	Flasks	N:F:M	Jarman et al.1978
DSM 576	.-.	4,98	Batch bioreactor	C.M.	Clementi et al. 1995
DSM 576	20g glucose	3.9	Batch bioreactor	C.M	Parente et al. 1998
Strain E	20g sucrose	2	Continuous Bioreactor	N.F.M	Annison & Couperwhite, 1986
Pseudomonas species					
P. syringae pv. glycinea					
Strain A-29-2	50g fructose	5	Flasks	C.M.	Fett & Wijey, 1995
P. pv. Pisi Race 2	50g fructose	5	Flasks	C.M.	Fett & Wijey, 1995
P. mendocina					
Strain UI	50g glucose	20	Batch Bioreactor	C.M.	Sengha et al. 1989
NCIB 10541	90g glucose	20	Continuous bioreactor	C.M.	Hacking et al. 1983

C.M. : Complex, not defined medium; N.F.M : Nitrogen free medium.

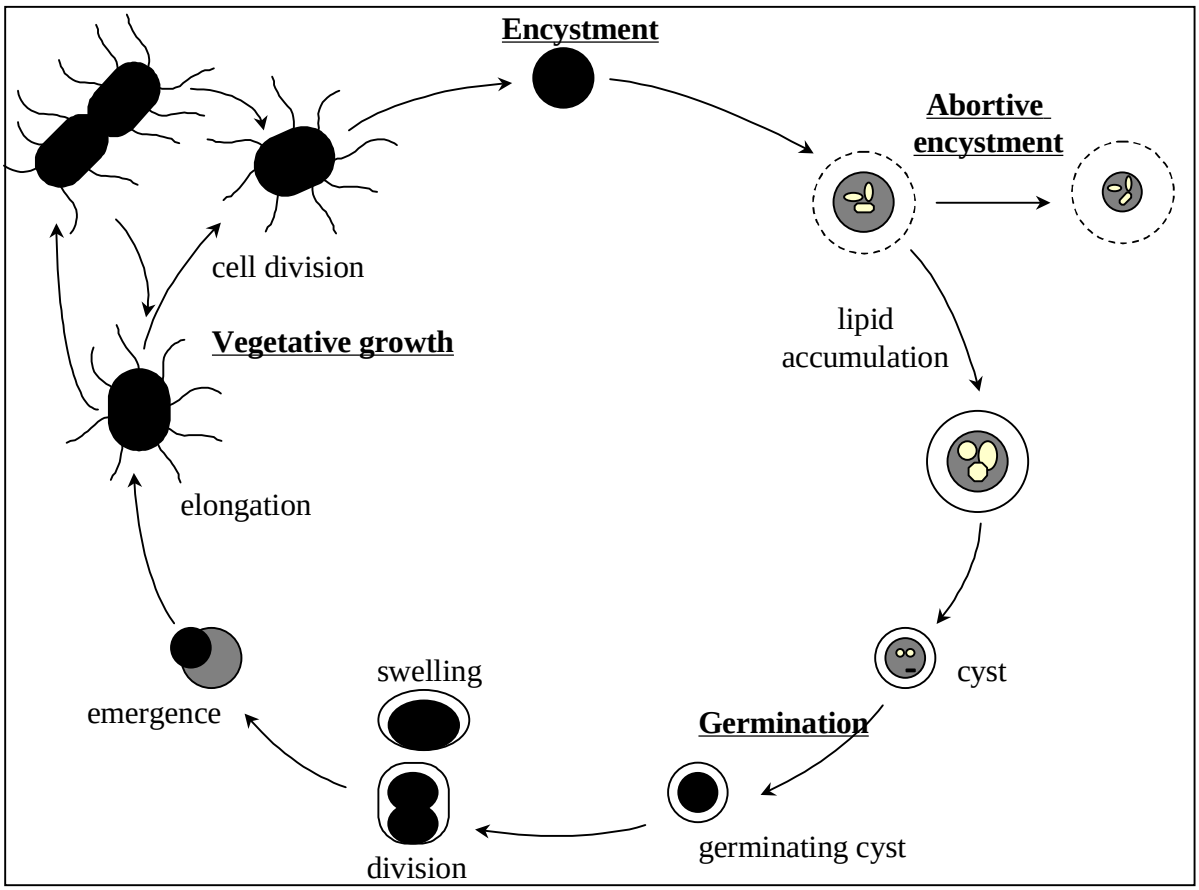


Fig. 2.3 Schematic diagram of the life cycle of *Azotobacter vinelandii* taken from Fyfe and Govan (1983).

Azotobacter cells grow well in media in which sucrose is the carbon source but the cells form few cysts. Cyst formation can be induced on nitrogen free agar medium which is supplemented with 0.2% butyl alcohol as the sole carbon source. It was also reported that substrates which enhance PHB production in cells promoted the encystment of *Azotobacter*. ß-Hydroxybutyrate, the monomer of poly-ß-hydroxybutyrate, a polymer known to accumulate prior to encystment, was found to induce the encystment in *Azotobacter* (Lin and Sadoff, 1968).

2.2.3. The biological function of alginate in both producing bacterial spp.

The role of exopolysacharides in nature has not been clearly established and is probably diverse and complex. It has been suggested that they may protect against dessication

phagocytosis and phage attack, participate in uptake of metal ions, as adhesive agents or ATP sinks or to be involved in interactions between plants and bacteria (Fyfe and Govan, 1983).

Alginates in phoeophyta are believed to act as a structure forming component providing mechanical strength and flexibility. This, however, explains the difference in alginate compositions in different algae or in different regions of the same alga.

Concerning the role of alginate for the pathogenic bacteria *P. aeruginosa*, the causative agent of cystic fibrosis, it serves to protect the bacteria from adversity in its surrounding and also enhances adhesion to solid surfaces. As a result, biofilm develops which is advantageous to the survival of the bacterium in the lung. The same strain, however, produces alginate lyase which cleaves the polymer into short oligosaccharides resulting in increased detachment of the bacteria away from the surface, allowing them to spread and colonize new sites (Boyd and Chakrabarty, 1995).

For alginate-producing *pseudomonads* (with the exception of *P. aeruginosa*) no natural ecological niche was reported. This suggests that in the majority of natural habitats alginate biosynthesis provides no advantage to the organism. However, alginate producing mutants of *P. fluorescencs*, *P. putida* and *P. mendocina* were isolated by selecting for antibiotic resistance (Govan et al. 1981).

Since exopolysaccharides are known to chelate heavy metals, it was also reported that alginate formation was induced, in plant-parasitic pseudomonads, by treatment with a bacteriocidal spray containing copper. Thus the secretion of alginate may contribute to some plant-bacterial diseases. Sodium chloride and ethanol were shown to significantly increase alginate production in a variety of fluorescent pseudomonads, suggesting that osmolarity and dehydration may be general signals for production of this polysaccharides (Kidambi et al. 1995). In general, alginate may contribute to a range of properties the specific roles of which might depend upon the environment.

In contrast to *Pseudomonas*, *A. vinelandii* is a stable producer of acetylated alginate under various conditions. Alginate production is a mandatory requirement for cyst formation in the nitrogen fixing bacterium. Polyguluronic acid rich polymer was found in the exine,

polymannuronic acid rich alginate was found in the central bodies of the cyst much more than in the intine and exine coats. This polysaccharide coating protects the cells from desiccation and mechanical stress, and cysts have been reported to survive in dry soil for several years. Under favourable conditions, including the presence of water, the alginate coating will swell and the cyst germinates.

The structural significance of alginate in the formation of microcyst does not explain the abundant production of alginate in metabolically active vegetative cells. Alginate does not serve as an overflow metabolite but rather that it *may* act as a protective barrier against heavy metal toxicity, as a barrier for diffusion (to oxygen), as an ion exchange system with enhanced selectivity for Ca^{2+}, or provide the bacterium with a hydrophilic, negatively charged coating which provides protection against attack and adverse environmental conditions (Fyfe and Govan, 1983). Recent studies revealed that alginate-encapsulated *Azotobacter chrococcum* were more protected against the depressive effect of phages than non-immobilised cells (Hammad, 1998).

It is therefore reasonable to believe that alginate has no single function for the vegetative cell itself, but rather provides the cell with a multitude of protective properties under various environmental conditions.

2.3. Physiology of alginate production

2.3.1. Pathway of biosynthesis of the precursor for polymerisation, GDP-mannuronic acid

The *A. vinelandii* and *P. aeruginosa* alginate-biosynthesis pathways are very similar (Lynn and Sokatch, 1984; Lioret et al. 1996) Exopolysaccharide is produced by *A. vinelandii* from a range of mono- and disaccharides including glucose, fructose, lactose, maltose and mannitol (Jarman, 1979).

Fig. 2.4 shows the different pathways in relation to alginate and PHB biosynthesis from sucrose in *A. vinelandii.*

Sucrose is first cleaved into glucose and fructose by the invertase enzyme. Based on NMR investigations to show the different carbohydrate fluxes into alginate biosynthesis in *A. vinelandii* (Beale and Foster, 1996), the metabolism of glucose is shown to follow the Entner-Doudoroff pathway. Fructose can be metabolised either to fructose-1,6-biphosphate or to mannose-6-phosphate which is further converted into GDP-mannuronic acid and then incorporated into alginate. No evidence was obtained for the involvement of GDP-guluronic acid as an alginate precursor. It is likely therefore that in *A. vinelandii* polymannuronic acid is the initial polymeric product. Then an extracellular polymannuronic acid C-5-epimerase enzyme catalyses the conversion of mannuronic acid in the polymer to guluronic acid residues. This enzyme seems to be dependant on calcium ions for activity (Larsen and Haug, 1971; Couperwhite and McCallum, 1974; Annison and Couperwhite, 1986). The level of calcium ions in the medium affects both the production and composition of the exopolymer. A lower calcium level in the growth medium results in the production of an exopolymer having a low content of guluronic acid (10-20%), whereas the proportion of guluronic acid becomes much higher when increasing the content of calcium in the growth medium (Skjak-Braek, 1992; Obika et al, 1993).

It is still not clear at what stage the acetyl groups were introduced into the polymer. Acetyl groups are always associated with the mannuronic acid residues and they were suggested to protect certain of these residues from the action of the epimerase enzyme (Fyfe and Govans, 1983).

On the other hand, the precursor of PHB biosynthesis is acetyl coA formed from catabolising the carbohydrate through the Entner-Doudorff pathway as shown in **Fig. 2.4.**

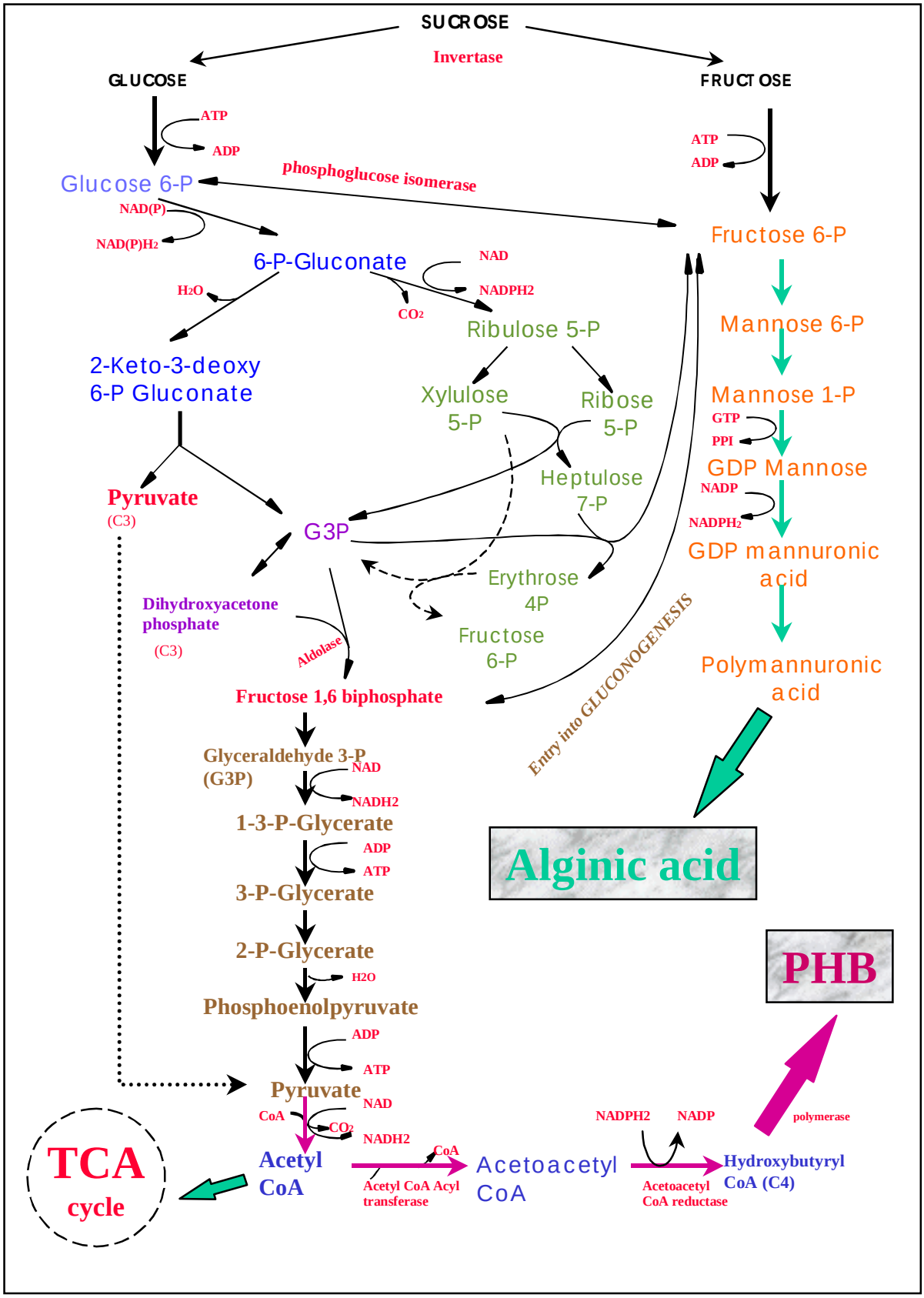


Fig. 2.4 Biosynthetic steps for the formation of alginate and PHB in *A. vinelandii*.

2.3.2. Alginate modifying enzymes

A number of different uronic acid-containing polysaccharides, including alginate, undergo post-polymerization modification to produce the final biologically active structure. The post-polymerization modification of alginate has a profound effect on the secondary structure and consequently on the biological role of this polymer (Gacesa, 1987).

Two enzyme systems are responsible for alginate modification, namely mannuronan C-5 epimerases and alginate lyase.

Mannuronan C-5 epimerase(s) is assumed to be located extracellularly in *Azotobacter vinelandii* whereas periplasmic in *Pseudomonas aeruginosa*. This enzyme has the ability to epimerize mannuronic acid to guluronic acid residues. This polymer level epimerization requires no known co-factor such as NADH/NAD^+. As already mentioned, the levels of calcium ions affect both the activity and reaction patterns. Alginate with a preponderance of mannuronic acid residues and with a homopolymeric structure is produced when growing with lower Ca^{+2} levels, while at high concentration of Ca^{+2} ions the epimerase will generate polymers with more guluronate residues and with high frequency of MG transitions. By limiting or increasing the degree of epimerisation the cells can form polymers with the ability to form soft flexible gels or hard brittle gels (**Fig. 2.5**).

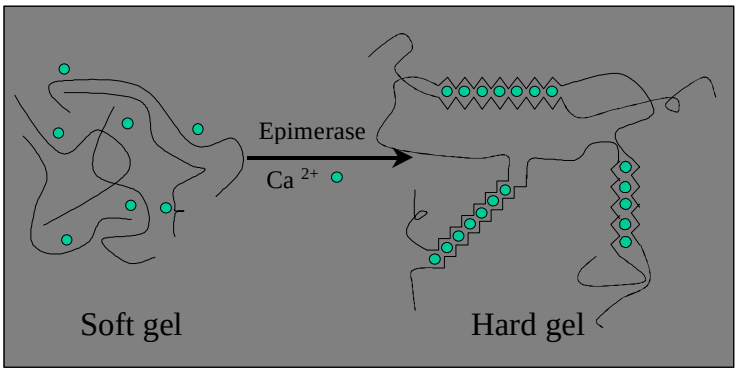


Fig 2.5 Proposed scheme of reaction during epimerization of alginate.

Alginate lyases, on the other hand, are located in the periplasm in both bacteria. Although there is no evidence that alginate serves as a carbon and energy reserve for *azotobacters* (they cannot use the polymers as sole carbon and energy source), these bacteria as well as *pseudomonads* have the ability to depolymerise alginate into smaller chains of oligomers by

alginate lyases (Ertesvag et al. 1998, Rehm, 1998). Since these enzymes can split alginate intramolecularly and since physico-chemical studies have revealed that the viscosity is directly proportional to the alginate molecular size, their secretion in the culture medium is of undesirable aim, if a good quality alginate is to be produced.

Gacesa (1987) suggested that alginate lyases and the epimerases which at the polymer level convert D-mannuronosyl residues to L-guluronosyl residues have a common mechanism of action. And this should allow for the conversion of lyases into epimerases and vice versa.

2.3.3. Genetics of alginate biosynthesis

The genetics of alginate biosynthesis was first studied in *Pseudomonas* (**Fig. 2.6**) and the genes involved in the synthesis of GDP-mannuronic acid have all been identified and characterised as follows:

algA encoding the phosphomannose isomerase and GDP-mannose pyrophosphorylase, *algC* encoding phosphomannomutase and *algD* encoding guanosine-diphosphomannose dehydrogenase.

The polymerization process, however, occur through proteins localised in the cytoplasmic membrane. *alg8*, *alg44* and *algX* are candidates for being subunits of the alginate polymerase.

The alginate modifying enzymes, acetylase (*algI*, *algJ* and *algF*), epimerase (*algG*) and lyase (*algL*), are mainly found in the periplasm in *P. aeruginosa*. It is worth noting that transacetylation of the mannuronic acid residues prevents these residues from being epimerized to guluronic acid residues. Thus the periplasmic acetylase indirectly controls the periplasmic epimerase activity on the alginate polymer in *P. aeruginosa*.

On the other hand, the epimerization process in *A. vinelandii* is much more complicated. A set of five strongly related Ca^{2+}-dependant epimerases (encoded by *algE1-algE5*) was found, compared to only one mannuronan C-5 epimerase in *Pseudomonads aeruginosa* (*algG*). Ertesvag et al. (1994, 1995) reported that AlgE4 introduces MG blocks into alginates (not

gel forming, analogous to AlgG in *P. aeruginosa*), while AlgE2 introduces G blocks (gel forming alginate). The remaining three epimerases (AlgE1, AlgE3 and AlgE5) catalyse the formation of different variants of these two main types of alginate. No periplasmic activity of epimerase isolated from *A. vinelandii* was observed and these enzymes were found extracellularly.

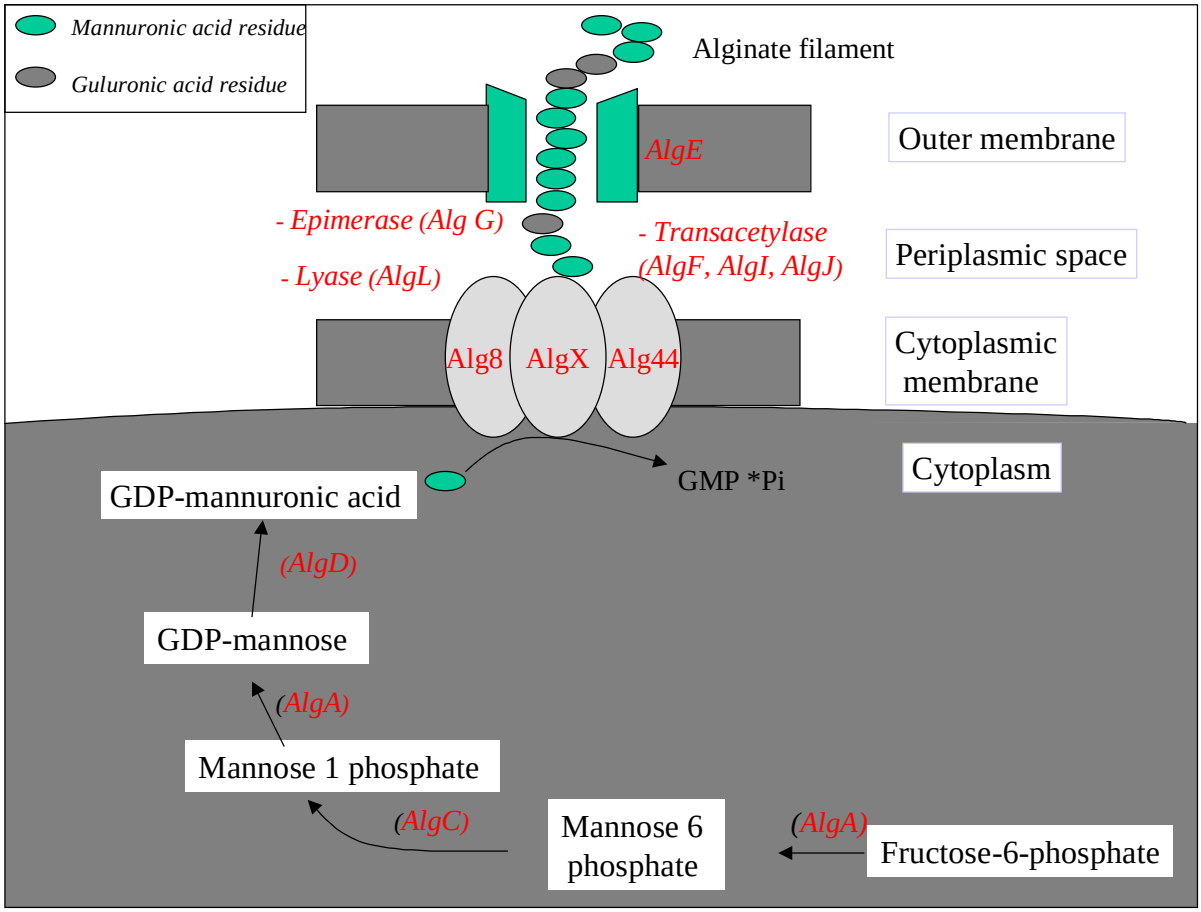


Fig. 2.6 Model of alginate biosynthesis, polymerisation, modification and export in *Pseudomonas aeruginosa* (after Rehm and Valla, 1997).

Putative equivalents of the *P. aeruginosa* *algL*, *algX*, *alg8*, *alg44*, *algD* and *algA* genes are present in *Azotobacter vinelandii* (Rehm and Valla, 1997).

The export of alginate is through an anion-selective protein (AlgE) in the outer membrane. Production of this protein (pore) is strictly correlated with the mucoid phenotype of *P. aeruginosa*. An *A. vinelandii* export gene (*algJ*) correspoding to *algE* in *P. aeruginosa* was recently identified (Rehm and Valla, 1997).

2.3.4. Nitrogen fixation and nitrogenase protection against oxygen stress

Among bacteria, aerobic nitrogen fixing microbes are rare. Aerobic nitrogen fixers are to be found mainly in the family Azotobacteriaceae which comprises the genera *Azotobacter*, *Azomonas*, *Beijerinchia* and *Derxia*. *Pseudomonas methanitrificans*, a methane oxidizing organism also fixes N_2 aerobically, *Mycobacterium flavum* and possibly one or two related species are also fixers. The remainder of the aerobic bacteria which can fix are facultative anaerobes which only fix N_2 when growing anaerobically. Anaerobic N_2 fixation is much more widely distributed (Postgate, 1971, 1974).

The nitrogenase enzyme complex, catalyzing the reduction of dinitrogen to ammonia is highly sensitive to oxygen. Since this enzyme is irreversibly inactivated by oxygen so that nitrogen fixation can be regarded as a strictly anaerobic process. This is not surprising if one considers that a strong reductant is required for nitrogen fixation. To keep the nitrogenase system anaerobic is no problem for strict anaerobes. Members of the diazotrophic azotobacters are able to grow under fully aerated conditions (Linkerhaegner and Oelze, 1995, 1997). However, It is worth mentioning that they do not fix optimally at high pO_2 values. They tend to be micro-aerophilic in the sense that, though they are obligate aerobes, their fixation is most effective at sub-atmospheric pO_2 values. Obligate aerobes as *A. vinelandii* employ two mechanisms for protection of the nitrogenase system against oxygen damage (Moshiri et al. 1995; Linkerhaegner and Oelze, 1997).

1- Respiratory protection. *Azotobacter* sp. possess a very active branched respiratory system (**Fig. 2.7**). At a high degree of aeration, *A. vinelandii* exhibits a very high respiration rate. The high oxygen uptake rate is associated with the de novo synthesis of both cytochrome *d* oxidase and NADH and NADPH dehydrogenases (Liu et al. 1995). Electrons from the primary dehydrogenases are channeled to a central quinone-cytochrome *b* region that exhibit proton translocation properties (site II).

At least two different branches of cytochromes can receive electrons from cytochrome *b*. The cytochrome *b*—*d* branch predominates when cells are growing diazotrophically at a high degree of aeration. This pathway is uncoupled from energy conservation and thus

allows rapid passage of electrons. It is this rapid oxidation which is thought to deplete cytoplasmic oxygen and thereby afford respiratory protection. When oxygen is low, more electrons are transferred from cytochrome *b* through cytochrome c_{4-5} to the cytochrome $o+a_1$ terminal oxidase (cyanide sensitive branch) which is coupled to ATP synthesis at site III. Increased respiration through cytochrome *d* was reported recently to enhance the microaerophilic nitrogen fixation in *Klebsiella pneumoniae* (Comaduran et al. 1998).

Thus with increasing oxygen concentrations the rate of respiration can be increased by a partial uncoupling and this renders the inside of the cell almost anaerobic. Although this is a waste of NADH but it does protect the nitrogenase against oxygen damage.

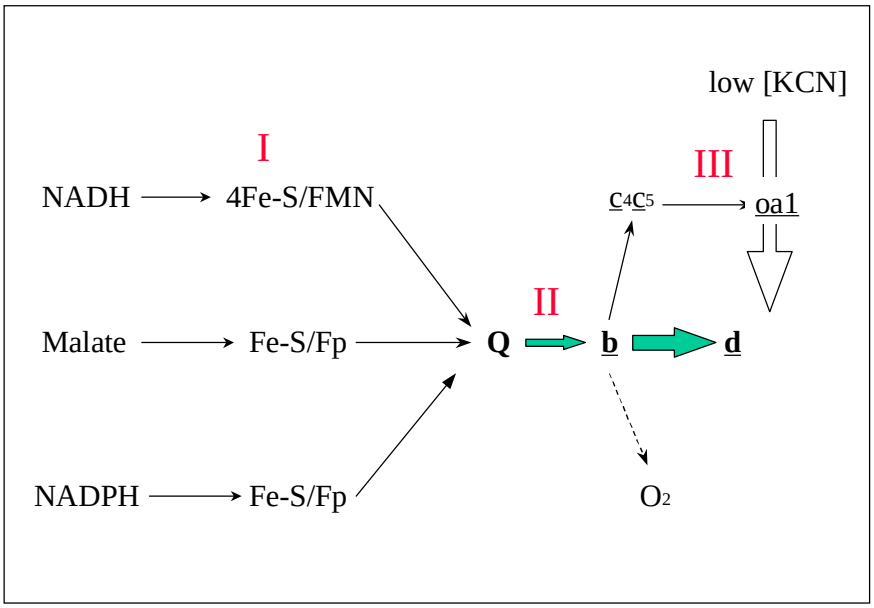


Fig. 2.7 Respiratory system of *A. vinelandii*. Solid arrows represent pathway of electron transfer, the broken arrow represents the cyanide-insensitive autooxidation of cytochrome b, and the open arrow represents the site of action of low concentrations of cyanide. I, II and III are the approximate sites of energy coupling (proton translocation) (after Haddock and Jones, 1977).

2- Conformational protection. In case of ineffective respiratory protection and following a sudden increase of oxygen concentration, the nitrogenase is switched off. Nitrogenase activity appears again after lowering the oxygen concentration. The enzyme is apparently protected by a conformational change and by the association of the protective protein. Moshiri et al. (1995) concluded that under periods of oxidative stress and to create an inactive but oxygen-stable nitrogenase complex, protective protein (FeSII) are then produced which confers conformational protection to nitrogenase by binding to the MoFe and Fe proteins. And when oxygen levels are favourable for nitrogenase function, the 'oxygen-stable nitrogenase complex' dissociates and the N_2-fixing process proceed without the need for *de novo* nitrogenase synthesis.

In most filamentous cyanobacteria the fixation of N_2 takes place in a special type of cell, the ***heterocyst.*** These cells are larger than the vegetative cells and are surrounded by a rather thick wall, are devoid of photosystem II and, therefore, cannot produce oxygen. Thus nitrogen fixation in this blue green algae takes place in almost anaerobic conditions (Gottschalk, 1988, Lichtl et al. 1997).

2.3.5. Poly-ß-hydroxybutyrate and carbon dioxide as major undesirable products

In any evaluation of any *A. vinelandii* strains for alginate production, it must be noted that substrate may be converted to exopolysaccharide, to the intracellular storage polymer poly-ß-hydroxybutyric acid (PHB) or wasted in respiration as CO_2. Their production constitute undesirable fates for carbon source when seeking to maximize alginate production.

The synthesis of PHB is associated with oxygen limitation and may comprise up to 70% of the cellular dry weight in certain strains of *Azotobacter vinelandii* (Brivonese and Sutherland, 1989; Page and Knosp, 1989; Chen and Page, 1994; Quagliano and Miyazaki, 1997; Bormann et al. 1998; Kim and Chang, 1998). Nitrogen fixing cultures form PHB efficiently only when they are oxygen limited. This condition presumably decreases the amount of NADPH consumed in the respiratory protection of nitrogenase and the cell tends to accumulate reducing equivalents which in turn inhibits citrate synthetase (Leonardon et al.

1995), this leads to the inactivation of the TCA cycle and thus most of the carbon is directed to PHB. PHB accumulation, therefore, serves not only as a store of carbon and energy but also as an electron sink into which excess reducing power can be channelled. When the bacterium encounter carbon limitation, these internal PHB reserves are then used as a source of carbon and energy to enable the cells to form cysts.

Furthermore, both PHB accumulation and alginate secretion are associated with the successful completion of the encystment process in starved *Azotobacter vinelandii* cells. Thus it may be difficult to separate the synthesis of these two polymers completely, rather to direct preferentially most of the carbon to the alginate biosynthetic pathway.

On the other hand, under oxygen saturation conditions respiration and biosynthesis become a more important electron sink than PHB production and again most of the carbon is directed to the TCA cycle, for nitrogenase protection, in an energy and carbon wasting mechanism.

Thus to secure a maximum yield of alginate, strict control of the oxygen supply is therefore vital, especially since the production of alginate itself hampers oxygen transfer by its effect on the viscosity of the medium.

2.4. Biotechnological optimisation of the microbial process for alginate production by *Azotobacter vinelandii*

2.4.1. Microaerophilic process and dissolved oxygen tensions control

A number of biomaterials including alcohols, amino acids, antibiotics, vitamins, vaccines and enzymes are effectively produced by microorganisms and animal cells at low concentrations of oxygen (microaerobic) or under oxygen limiting conditions.

With few exceptions, there is a general agreement among the investigators that the oxygen supply is of critical importance for alginate production by this bacterium especially when grown diazotrophically. Dawes (1990) stated that in phosphate limited continuous culture the amount of exopolysaccharide produced by *A. vinelandii* is dependant on both the DOT and

the carbon source. He concluded that in the range of 1-5% DOT the alginate synthesis remains constant while it decrease above and below this values.

Therefore, precise control for pO_2 is required to allow a clear interpretation of the experimental results, to better understand the metabolism, and to optimize the alginate production process.

Since the strict control of dissolved oxygen tension at low levels (< 5% air saturation) without altering the mixing pattern and fluid dynamics is difficult to achieve in laboratory bioreactor (Diaz et al. 1996), with few exception, investigations for alginate production by *A. vinelandii* were carried out in pO_2 uncontrolled systems or with the manual or automatic help of the stirring rate (Jarman et al. 1978; Tsai et al. 1979; Annison and Couperwhite, 1986 a,b; Brivonese and Sutherland, 1989; Savalgi and Savalgi, 1992; Clementi et al. 1995; Beale and Foster, 1996; Pena et al. 1997; Parente et al, 1998)

2.4.2. Non-Newtonien alginate fluids and the role of fermentor hydrodynamics

In aerobic submerged exocellular microbial polysaccharides fermentations such as xanthan, dextran and alginate, oxygen supply to the media and mass transfer of O_2 to growing microbial cells is still a major technical problem affecting microbial productivity, since the solution becomes highly viscous and non-Newtonian during fermentation owing to the presence of polysaccharides in the culture medium. The relationship between introduced mixing energy and the oxygen transfer rate in solutions with pseudoplastic behaviour is still difficult to establish (Dussap and Gros, 1985). In case of alginate production by *A. vinelandii*, the low oxygen solubility in fermentation media coupled with high O_2 consumption rate (reported for *Azotobacter*) make the task of O_2 supply (aeration) difficult. On the other hand, increasing the mechanical stirring to enhance the aeration, may cause strong turbulence in local zones where damaging of the sensitive cell membrane occurs (Toma et al. 1991).

Changing the rheology of the fluid will obviously affect the flow pattern created by the impeller. Around the impeller the fluid is turbulent, and turns to be laminar or stagnent when the shear stresses are below the yield stress of the polymer. The impeller creates a cavern in

which the fluid is moving relatively fast and where the flow is turbulant. However, in the bulk of the fluid, where the shear stresses are below the yield stresses, the fluid is not moving at all (Bakker and Gates, 1995). Literature reports on the effect of excess turbulence -with controlled dissolved oxygen concentration- on alginate production and viability of *A. vinelandii* are still missing.

2.4.3. Medium formulation

Beside the paramount role of aeration in determining the yield of alginate from *A. vinelandii*, there has been many attempts to increase alginate productivity through medium formulation.

The addition of fixed nitrogenous compounds to the culture medium was reported to be beneficial or detrimental for alginate production by this bacterium. Brivonese and Sutherland (1989) concluded that varying the source of peptone used in the medium could alter alginate yield by up to 30%, suggesting a more specific role for nitrogenous nutrients.

The effect of phosphate on alginate production in *A. vinelandii* was rather controversially reported in the literature. Horan et al. (1981) observed a drop in alginate yield in a medium with excess phosphate and for their continuous culture studies a phosphate limitation gave maximum alginate production. Brivonese and Sutherland (1989), on the other hand, reported that phosphate might simply act to buffer the medium which will tend to become more acidic as alginate is secreted and used a phosphate rich medium (7.5 g K_2HPO_4). Precise and quantitative studies in a pH and pO_2 controlled bioreactor are still needed.

The effect of different growth limitations on alginate production has also been investigated. Molybdate limitation followed by phosphate or iron limitation, the condition routinely used, gave the highest specific rates for alginate production. Surprisingly, under sucrose limitation (in continuous culture), a condition where the cell would be expected to make the most efficient use possible of its available carbon and energy substrate, alginate was produced at similar rates to other limitation (Deavin et al. 1977). However, the same author concluded that the specific alginate formation rate ($q_{alginate}$) does not vary greatly with changes in the cultural compositions. Using a composite design experiment in shake flask, Clementi et al.

(1995) elucidated that alginate production in *A. vinelandii* was unaffected by most environmental conditions.

With respect to alginate biosynthesis in batch culture, Deavin et al. (1977) observed that alginate synthesis paralleled growth. In contrast to this Horan et al. (1981); Chen et al. (1985), and Brivonese and Sutherland (1989) observed that alginate secretion continues after growth had ceased. Parente et al. (1998), on the other hand, concluded that alginate production was growth associated at 5% dissolved oxygen tension but significant amounts of alginate were produced after growth had stopped at a lower DOT value.

Contradictory results were also obtained on the effect of growth rates on alginate production. Jarman (1979) observed that the specific rate of alginate synthesis from sucrose was independant of specific growth rate, a situation also noted by Fyfe and Govan (1983). On the other hand, Parente et al. (1998) recently reported that the relation between specific alginate production rate and specific growth rate was non linear and dependant on the DOT.

3. MATERIALS, METHODS AND CALCULATIONS

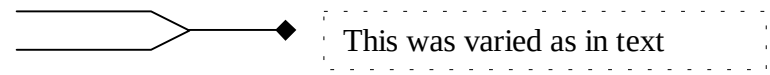
3.1 Microorganism

Azotobacter vinelandii (Egyptian isolate, DSM 93-541b) was used in this study. Stock cultures were maintained on Jarman nitrogen free broth supplemented with 30-40% gylcerin and kept at -18°C. Short term viable cultures were maintained on Jarman agar plates at 4°C. Cultures used in the experimental studies were not older than 5 weeks.

3.2 Culture media

The synthetic nitrogen free Jarman medium having the following composition per liter of deionized water was used:

40	g	sucrose
0.4	g	$MgSO_4 . 7H_2O$
0.4	g	NaCl
160	mg	KH_2PO_4
640	mg	K_2HPO_4
84	mg	$CaCl_2 . 2H_2O$
2	mg	$NaMoO_4 . 2H_2O$
6	mg	$FeSO_4 . 7H_2O$
2.9	mg	H_3BO_4
1.2	mg	$CoSO_4$
0.1	mg	$CuSO_4 . 5H_2O$
0.09	mg	$MnCl_2 . 4H_2O$
1.2	mg	$ZnSO_4 . 7H_2O$



Sucrose, $MgSO_4$, $CaCl_2$ and phosphate mixtures were separated from the other media components during sterilisation. The $FeSO_4$ solution was sterilised by filtration using Millipore filter 0.2µm.

3.3. Cultivation system

3.3.1. Cultivation in shake flask

Flask experiments were done in 250 ml baffeled Erlenmeyer flasks containing 50 ml of the above medium and kept on a rotary shaker at 200rpm at 30°C. The pH was initially adjusted at 7.3 which decreased during the fermentation time due to alginate production. The inoculum was always set to contribute to 10% (v/v) of the experimental volume.

3.3.2. Cultivation in bioreactor

Fig. 3.1 shows a schematic diagram of the bioreactor set-up and the control units used throughout this study. They are described in detail in the following sections.

3.3.2.1. Reactor

Fermentations were carried out in a 5l stirred tank bioreactor (Biostat B, B-Braun Biotechnolgia, Germany) with a working volume of 2.5-3.5 liters. The bioreactor was equipped with temperature, pH, pO_2 and agitation speed measure and control unit which was connected to a process computer (**UBICON**: Universal Bioprocess Control System, GBF, Braunschweig, Germany).

3.3.2.2. Agitation

The bioreactor has a stirrer shaft with three six-bladed disk impellers. Agitation was controlled in the range of 200-1000rpm with a PID controller installed in the control unit of B-Braun.

3.3.2.3. Temperature

Temperature was measured by a Pt-100 temperature sensor and controlled at 32°C by single loop PID controller. The thermostat system is an open, pressure free system. It includes an electric heater and a valve for cooling water supply. Circulation pump delivers water of the preadjusted temperature to the culture vessel. The digital measurement and control system ensures a precise and constant temperature control.

3.3.2.4. pH

The pH was measured by an autoclavable pH electrode (Ingold) and kept at 7.2 +/- 0.2 by PID cascade controller supplied in the control unit of the reactor. This was done by the automatic addition of HCl (0.1M) or NaOH (0.1M) by acid and base pumps in the control

unit of B-Braun. The electrode was calibrated before sterilisation and checked afterwards during sampling.

3.3.2.5. Aeration and pO_2

The bioreactor was aerated with a fine-porous gas distributor. The inlet gas was sterilised with membrane filter. The measurement of the dissolved oxygen (pO_2) was done by an autocolavable pO_2 electrode (Ingold). The electrode was calibrated after sterilisation using nitrogen and air. A thermal mass flowmeter/controller was used to verify the aeration rate. The total aeration rate was controlled at constant value (2 l/min) by UBICON. The pO_2 was controlled in the range of 0-10% with the accuracy of 1% by mixing nitrogen and air in the inlet gas by PID controller defined through the UBICON facilities.

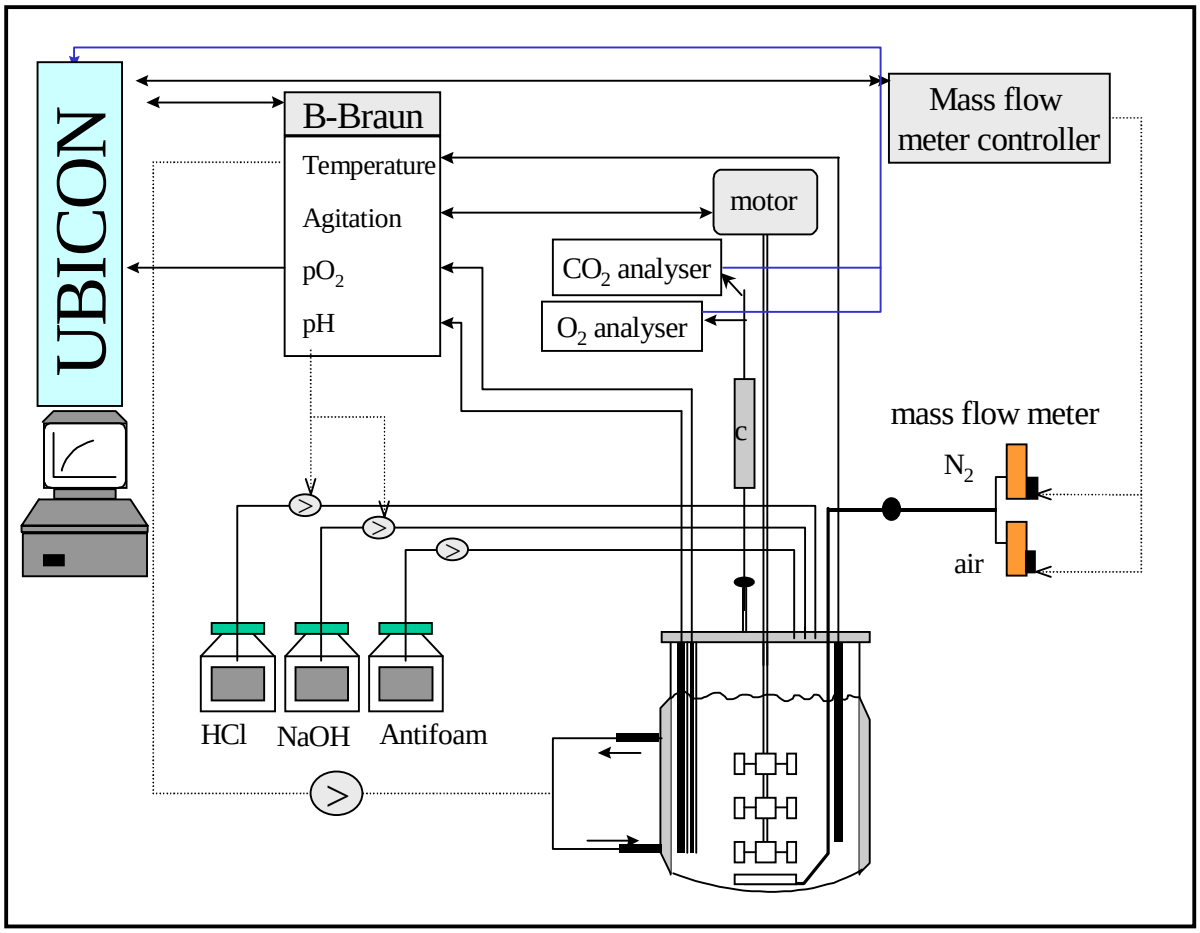


Fig. 3.1. Schematic diagram of B- Braun fermentor used throughout this work.

3.3.2.6. Foam control

Foam was controlled manually by the addition of the antifoam agent ‘Desmophen‘ (1% v/v sterile solution) to the bioreactor medium.

3.3.2.7. Volume and weight control

Batch and fed batch fermentations were started with a defined initial volume. In continuous fermentations, however, the bioreactor was fed continuously with fresh medium and the volume of the culture had to be controlled at a distinct value.

Alginate acts as a surface active agent **(**Ghildyal et al. 1988**)** causing the preferential removal of alginate from medium into the foam. This was observed as the biomass concentration in foam was lower than inside the medium. Therefore, establishing a steady state in chemostat culture using only the level tube was unsuccessful and, either a combination between level tube and peristaltic pump or a calibrated balance connected to a withdrawing peristaltic pump were used to obtain a steady state culture as indicated in **Fig. 3.2.**

Whenever operating conditions were changed in continuous culture, at least four residence times were allowed to pass before steady state parameters were assessed.

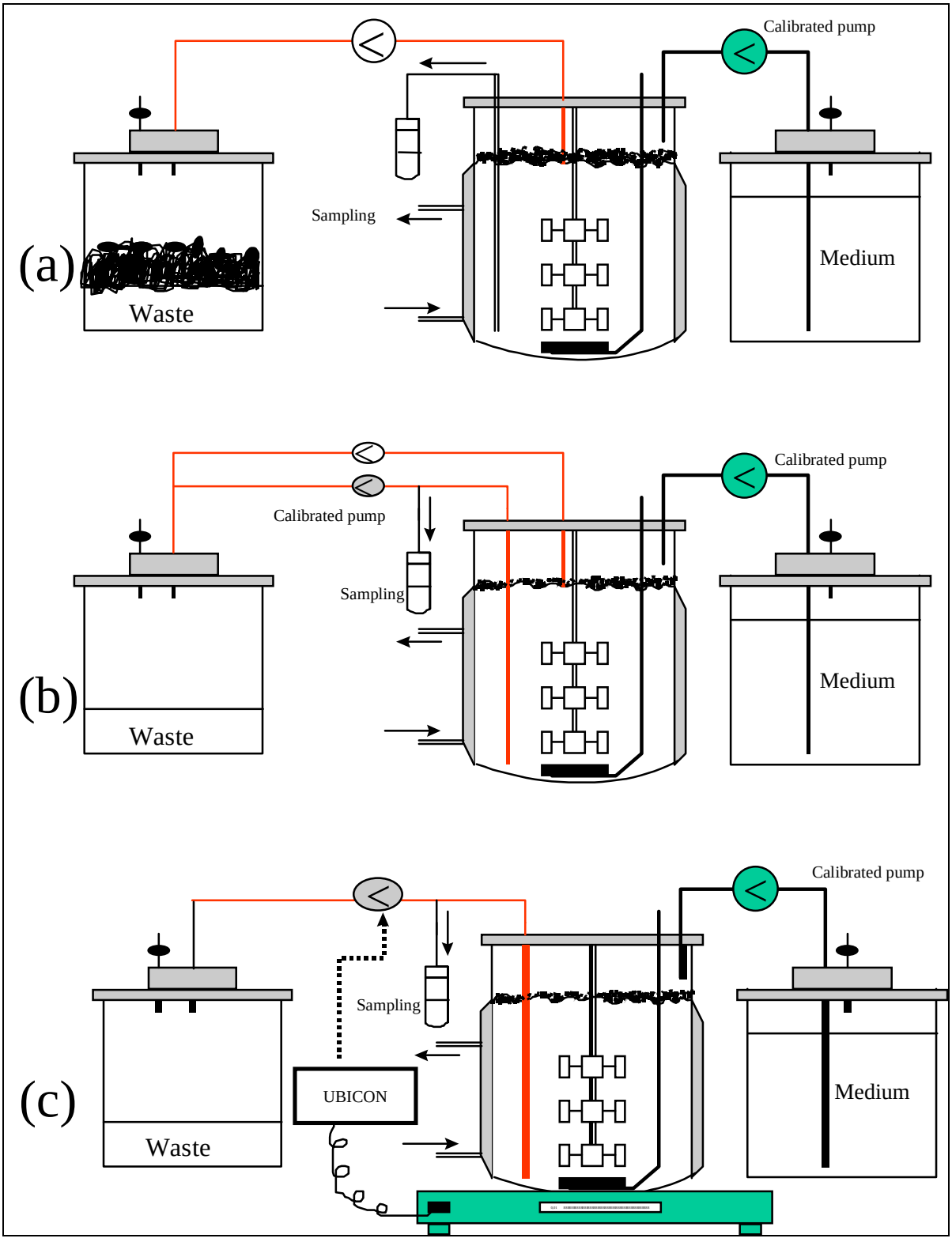


Fig. 3.2 Experimental set-up of chemostat culture. (a) the level tube withdraws only foam and no steady state was obtained, (b) level tube withdraws only the slight difference between the inlet and outlet pump and (c) use of balance connected with harvesting pump controlled through UBICON

3.4. Analytical methods

A flow diagram to demonstrate the steps of the experimental work is presented in **Fig. 3.3.**

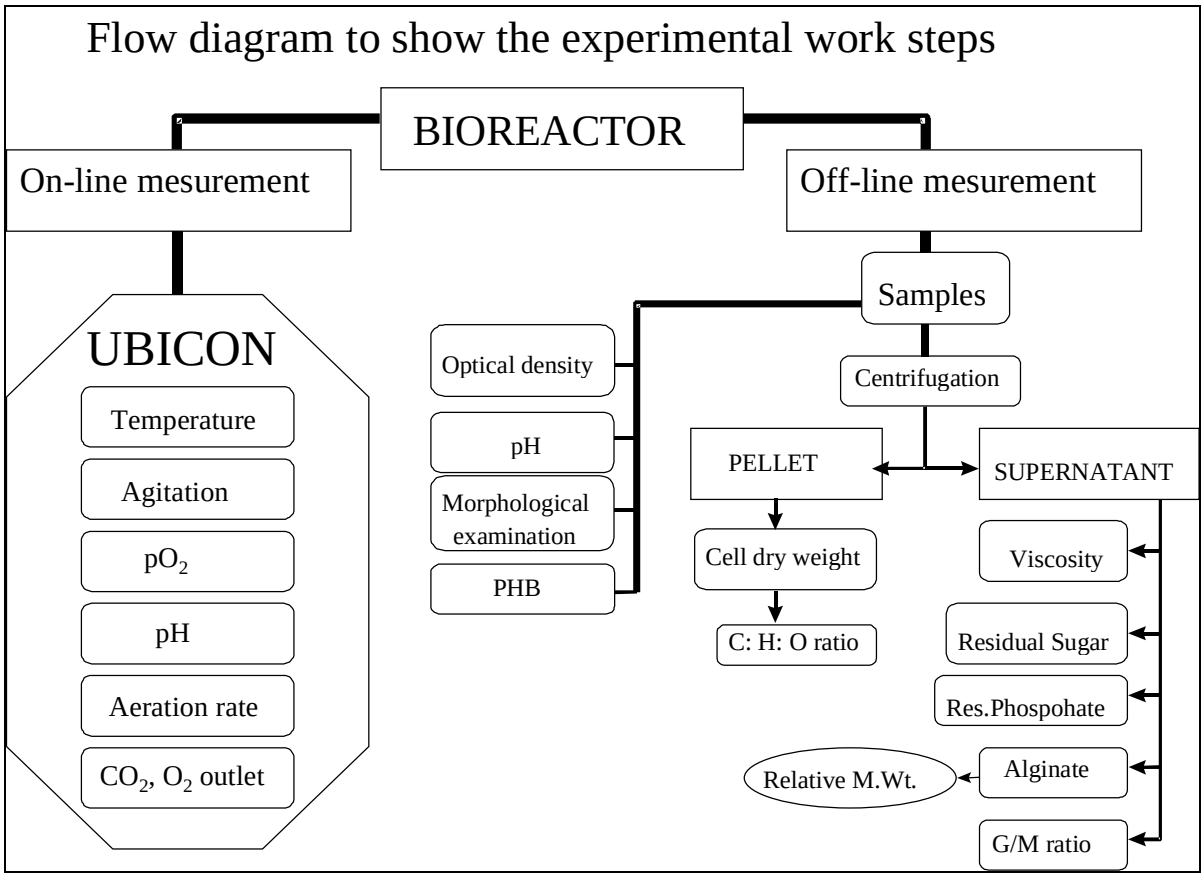


Fig 3.3 Diagrammatic representation of experimental work steps.

3.4.1. Optical density

The optical density (OD) of the culture was measured at 600 nm with a Pharmacia Biotech Spectrophotometer. The samples were adequately diluted when the OD value exceeded 0.6.

3.4.2. Biomass and alginate determination

Biomass and alginate dry weight were determined by gravimetrical methods as follows:
1ml of 0.5M EDTA-sodium salt and 0.5ml of 5M NaCl were added to 25ml sample of culture broth to separate the capsular alginate. After stirring for 5min the sample was centrifuged at 18000 rpm at 20°C for 30min to precipitate the cells. The cells were washed twice with

destilled water, recentrifuged and then dried at 80°C for 24h. The supernatant was cooled and alginate was then precipitated by the addition of 3-volumes of ice cold isopropanol which was then recovered by centrifugation at 18000 rpm at 4°C for 30min. The precipitate was dissolved in water, precipitated again, centrifuged and then finally dried at 80°C for 24h. For each determination at least two samples were used.

The relation between optical density and biomass dry weight is shown in **Fig 3.4**.

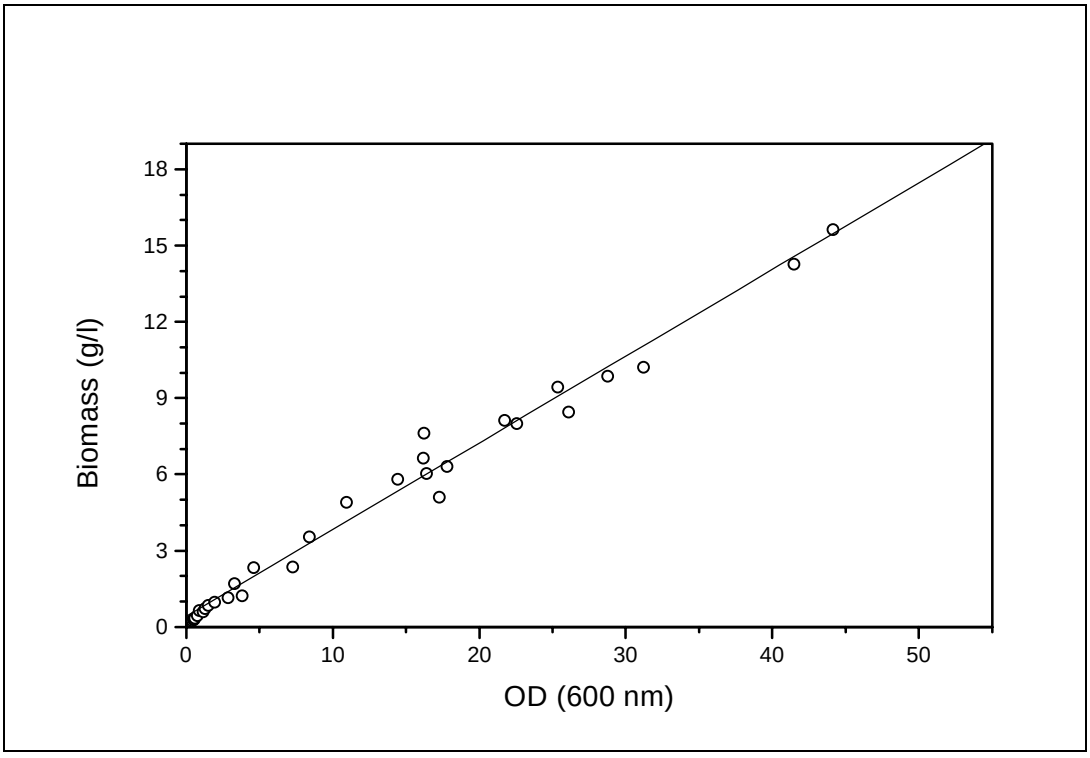


Fig 3.4 Correlation between optical density and biomass dry weight

3.4.3. Poly-ß-hydroxybutyrate determination

The PHB content in the cells was determined according to the method of Senior et al. (1972) as follows:

A sample of culture solution (0.1 – 0.2ml) was added to 4ml of 10% alkaline hypochlorite solution and then left for 1 day. 0,4ml of the sample were centrifuged for 30min to precipitate the PHB. The solid pellet was resuspended and washed with 1ml portions of water, acetone and ether. Chloroform was added and allowed to boil in a water bath at 100°C. The settled material after evaporation of chloroform was dried at 40° C for 30min.

The white powder was dissolved in concentrated H_2SO_4 (2.5ml) and heated for 10min at 100°C. After cooling, the solution was measured photometrically at 235nm against H_2SO_4 blank

A standard curve was established with PHB concentrations ranging from 2 - 20 µg/ml PHB.

3.4.4. Enzymatic determination of sucrose

Sucrose as well as fructose and glucose were determined with an ultraviolet test-kit combination measured at a wave length of 340nm (Boehringer Mannheim, Germany). The determination of sucrose, glucose and fructose is based on the formation of $NADH_2$ measured by the increase in absorbance at 340 nm.

3.4.5. Phosphate determination

Phosphate was determined colorimetry by the method of Boltz (1972) as follows:
the following two reagents were prepared
Solution a: 0.88g ascorbic acid + 1.25ml acetone and then complete the mixture to 100 ml with distilled water
Solution b : 0.5g ammonium heptamolybdate + 3.1ml concentrated sulphuric acid and then the mixture was completed to 100ml with distilled water.

In an eppendorf tube 0.5ml of 'solution a' was mixed with 0.5ml 'solution b' and 0.25ml cell free sample, then heated at 60°C for 10 min in a water bath. Stopping the reaction was then done in an ice bath and the intensity of the blue-colour produced was then measured at 660 nm.
A calibration curve was done using different known concentration of potassium phosphate with water as a blank sample.

3.4.6. Guluronic / mannuronic acid ratio

The ratio between guluronic and mannuronic acid units was estimated by the calorimetric reaction of carbazole according to the method of Knutson and Jeanes (1968) as follows:.

For 50µg alginate the reactivity ratio were calculated according to **Eq. 3.1.**:

$$R = \frac{A'_{mix}}{A_{mix}} = \frac{f_1 A'_1 + (1-f_1) A'_2}{f_1 A_1 + (1-f_1) A_2} \qquad [3.1]$$

where

A'_{mix} is the absorbance of 50µg alginate sample without borate at 55°C

A_{mix} is the absornace of 50µg alginate sample with borate at 55°C

f_1 is the fraction of the first uronic acid (mannuronic acid) in the alginate sample

f_2 is the fraction of guluronic acid in the alginate sample

A_1 and A_2 are the absorbance of 50µg of mannuronic acid and guluronic acid without borate at 55°C.

A_1' and A_2' are the absorbance of 50µg of mannuronic acid and guluronic acid with borate at 55°C.

from the above equation

$$f_1 = \frac{A'_2 - A_2 R}{R (A_1 - A_2) - A'_1 + A'_2} \qquad [3.2]$$

and thus

$$\text{mannuronic acid fraction} = f_1 * 100 \qquad [3.3]$$

$$\text{guluronic acid fraction} = (1 - f_1) * 100 \qquad [3.4]$$

3.4.6.1. Reaction of alginate sample without borate

0.1 ml of 0.5g/l alginate sample was mixed with 1ml of concentrated sulphuric acid and cooled in an ice bath, then mixed and heated at 55°C. After 20 min the sample was cooled in an ice bath and 30µl carbazole reagent (0.1% ethanol) was then added and allowed to stand for 3h. The colour was stable for 2h.

3.4.6.2. Reaction of alginate with borate

0.1 ml of 0.5g/l alginate sample was mixed with 1ml of borate sulphuric acid reagent (10mM H_3BO_3 in concentrated sulphuric acid) and cooled in an ice bath. 30µl of carbazole reagent was then added, mixed and heated at 55°C for 30min. Colour was stable for 2h at room temperature.
Standard curves of mannuronic acid as well as guluronic acid were made for the two reaction conditions (with and without borate)

3.4.7. Effluent gas

The content of carbon dioxide and oxygen in the effluent gas was analysed by a paramagnetic oxygen analyser (Oxygor, Maihak, Germany) and an infrared carbon dioxide analyser (UNOR, Miahak, Germany). Both gas analysers were calibrated using a reference gas mixture of known composition (15.9% O_2 :5% CO_2 and the rest was N_2) before and during fermentation run. Both gas analysers were connected to the UBICON. Oxygen uptake rate, carbon dioxide production rate and the respiratory quotient (RQ) were calculated online using the following equations (**Eq. 3.5 , 3.6 ,** and **3.7)**

$$Q_{CO2} = \frac{V_G'}{V_N}\left(X_{CO2}^{out}\left(\frac{1-X_{CO2}^{in}-X_{O2}^{in}}{1-X_{CO2}^{out}-X_{O2}^{out}}\right) - X_{CO2}^{in}\right) \quad [3.5]$$

$$Q_{O2} = \frac{V_G'}{V_N}\left(X_{O2}^{in} - X_{O2}^{out}\left(\frac{1-X_{CO"}^{in}-X_{O2}^{in}}{1-X_{CO2}^{out}-X_{O2}^{out}}\right)\right) \quad [3.6]$$

&

$$RQ = \frac{Q_{CO2}}{Q_{O2}} \quad [3.7]$$

where

V_G' = Total aeration rate (l h^{-1})

V_N = Molar volume of ideal gases = 24.45 (l mol^{-1})

X_{CO2}^{in} = Moler fraction of CO_2 in inlet gas mixture (mol mol^{-1})

X_{CO2}^{out} = Moler fraction of CO_2 in outlet gas mixture (mol mol^{-1})

X_{O2}^{in} = Moler fraction of O_2 in inlet gas mixture (mol mol^{-1})

X_{CO2}^{out} = Moler fraction of O_2 in outlet gas mixture (mol mol^{-1})

3.4.8. Relative molecular weight determination

The relative molecular weight was determined by gel permeation-high pressure liquid chromatography as follows: the mobile phase used was 0.1M phosphate buffer (pH 6.9) applying two TSK gel columns (TSK G6000PWHR followed by TSK G5000PWHR) arranged in rows. The signal was then detected by a differential refractive index detector (Beckman model 156). The columns were calibrated by broad dextran standards. Before applying to the columns the alginate samples (0.5g/l) were prepared with the purified alginate in the same eluting buffer and filtered through a 1.2µm pore size Millipore membrane to remove cellular debris. As no absolute molecular weight standards were available, gel chromatography only allowed to establish the relative dispersion of the polymer molecular weight.

3.4.9. Determination of rheological properties

The flow behaviour of alginate solution was studied at 30°C with a concentric cylinder viscometer at different shear rates and the rheological indexes K and n were calculated by a software (Mettler control-evaluation software SWR37) connected to the viscometer. The Ostwald and Wale model (power low: Eq. 3.8) was used to calculate those parameters.

$$\tau = K\,\gamma^{n} \qquad [3.8]$$

where τ = shear stress (pa)

K = consistency index ($mPas^{n}$)

γ = shear rate (s^{-1})

n = flow behaviour index

3.4.10. Elemental analysis

The elemental composition C H N of dried biomass and alginate was analysed using a PE 2500 series II CHNSO analyser Fa. Perkin Elmer. This analyser uses a combustion method to convert the sample elements to simple gasses (CO_2, H_2O and N_2). The resulting gasses are homogenized and controlled to exact conditions of pressure, temperature and volume. The homogenized gasses are allowed to depressurize through a column where they are separated in a step-wise steady-state manner and detected as a function of their thermal conductivities. Before the measurements, the system was calibrated using known standards, each sample was measured at least three times.

3.4.11. Material balance

Considering that the carbon source is converted mainly into biomass, alginate, PHB and carbon dioxide, in chemostat culture carbon balance was estimated using Eq. 3.9.

$$C_{rec} = \frac{w^{c}_{X} \cdot Q_{X} + w^{c}_{alg} \cdot Q_{Alg} + w^{c}_{PHB} \cdot Q_{PHB} + w^{c}_{CO_2} \cdot Q_{CO_2}}{w^{c}_{sucrose} \cdot Q_{S}} \qquad [3.9]$$

where,

C_{rec} is the carbon recovery (g/g). w^{C}_{X}, w^{C}_{alg}, w^{C}_{PHB}, $w^{C}_{CO_2}$ and $w^{C}_{sucrose}$ are the carbon contents of biomass, alginate, poly-ß-hydroxybutyrate, carbon diouxide and sucrose, respectively. Q_x, Q_{Alg}, Q_{PHB}, Q_{CO2}, and Q_S are the production and consumption rates of biomass, alginate, poly-ß-hydroxybutyrate, CO_2 and sucrose, respectively.

3.4.12. Morphological observation

3.4.12.1. Light microscopy

The morphological study of the cell as well as the slime layer characterisation were done by negatively staining the cell with 7% (w/v) aqueous nigrosin and were then visualised by dark phase microscopy. Capsules appear as clear zones around the refractile organism against a blue-black background.

3.4.12.2. Electron microscopy

3.4.12.2.1. Surface view preparation

Transmission electron microscopy was used to investigate the alginate concentration gradient around the cells. samples were picked up with carbon-coated collodion grids. The grids were plotted with filter paper, and alginate was positively contrasted by incubation on freshly prepared 1% aqueous ruthenium red solution for 1-2 min at room temperature. The grids were washed three times with distilled water by paper blotting, taking care not to let the surface fall dry. Finally the cells were negatively stained with 1% uranyl acetate for 10s, blotted, and air dried. Electron microscopy was done with a Zeiss EM 10B at 80kV with a magnification between 16,000 and 25,000.

3.4.12.2.2. Thin section preparation

Azotobacter vinelandii cells were preincubated in 0.5% (w/v) ruthenium red for 30 min at ambient temperature in the growth medium. Fixation was done by addition of 25% (v/v) glutardialdehyde to a final concentration of 1.25% (v/v) for 72h at ambient temperature and at 4°C. After centrifugation the fixed cells were resuspended in 0.1M

cacodylate buffer, pH 7.2 and washed by three sedimentation/resuspension cycles for 10 min each at room temperature. Washed cells were immobilized in 0.1M cacodylate pH 7.2 -buffered 2% (w/v) agar and were finally fixed with 1% OsO4 – 0.1M cacodylate pH 7.2 over night at 4°C. Cells were dehydrated on ice with an acetone serie and embedded in epoxy-resin (Spurr, 1969). Ultrathin sections (120 nm thickness) were poststained with lead citrate, according to Reynolds (1963), and were analysed with an CEM 902 Zeiss transmission electron microscopy at 80 kV in the range from x 12000 to x 20000 primary magnification.

3.5. Calculation of the process parameters

- In batch culture

*** Production and formation rate calculations.**

Growth rate was calculated as follows:

$$\mu = \frac{\ln(m_X^{t2} - m_X^{t1})}{(t_2 - t_1)} \qquad [3.10]$$

Similarly alginate and PHB production rate Q_{alg} and Q_{PHB} were calculated from the following two equations:

$$Q_{alg} = \frac{(m_{Alg}^{t2} - m_{Alg}^{t1})}{(t_2 - t_1)} \qquad [3.11]$$

$$Q_{PHB} = \frac{(m_{PHB}^{t2} - m_{PHB}^{t1})}{(t_2 - t_1)} \qquad [3.12]$$

*** Specific rate calculation.**

Specific sugar consumption rate was calculated with the help of the software Origin 4. (Microsoft). First both sugar and biomass concentrations were described polynomially as a

function of time. For the small time interval t, q_S can then be calculated from the following equation.

$$q_S(t) = \frac{dC_S(t)}{dt} \cdot \frac{1}{X(t)} \qquad (g\ g^{-1}\ h^{-1}) \qquad [3.13]$$

Similarly specific alginate and PHB production rate were described by the following two equations.

$$q_{alg}(t) = \frac{dC_{Alg}(t)}{dt} \cdot \frac{1}{X(t)} \qquad (g\ g^{-1}\ h^{-1}) \qquad [3.14]$$

$$q_{PHB}(t) = \frac{dC_{PHB}(t)}{dt} \cdot \frac{1}{X(t)} \qquad (g\ g^{-1}\ h^{-1}) \qquad [3.15]$$

- In chemostat culture.

*** Dilution rate (D) and specific growth rate (μ)**

the dilution rate D of continuous culture was calculated as follows:

$$D = \frac{F_m + F_{NaOH}}{V_L} \qquad (h^{-1}) \qquad [3.16]$$

where F_m is the medium flow rate (l/h), F_{NaOH} the consumption rate of NaOH (l/h) and V_L is the working volume of the bioreactor.

In chemostat culture the specific growth rate (μ) is identical with the dilution rate, provided steady-state conditions have been achieved (Pirt 1985).

$$\mu = D \qquad [3.17]$$

*** Specific rates and yield parameters.**

The specific sucrose consumption rate q_S was calculated from the following equation:

$$q_S = \frac{D \cdot (S_F - S_R)}{X} \qquad (g\ g^{-1} h^{-1}) \qquad [3.18]$$

where S_F is the sugar concentration in the feed and S_R the residual sugar concentration in the culture medium. The biomass dry weight concentration without PHB material (X) was estimated from the following formula

$$X = X_{total} - C_{PHB} \qquad (g\ l^{-1}) \qquad [3.19]$$

where C_{PHB} is the concentration of PHB in the culture medium, X_{total} is the biomass dry weight (with PHB).

The specific formation rate of PHB and alginic acid (q_{PHB}, and q_{Alg}) were calculated with the following formulas

$$q_{PHB} = \frac{D \cdot C_{PHB}}{X} \qquad (g\ g^{-1}\ h^{-1}) \qquad [3.20]$$

$$q_{Alg} = \frac{D \cdot C_{Alg}}{X} \qquad (g\ g^{-1}\ h^{-1}) \qquad [3.21]$$

where C_{Alg} is the concentrations of alginate in the culture supernatant as well as the capsular alginate.

The specific oxygen uptake rate and specific CO_2 production rate were calculated by the equations

$$q_{O2} = \frac{Q_{O2}}{X} \qquad (mmol\ g^{-1}h^{-1}\ \text{ or } g\ g^{-1}h^{-1}) \qquad [3.22]$$

$$q_{CO2} = \frac{Q_{CO2}}{X} \qquad (mmol\ g^{-1}h^{-1}\ \text{ or } g\ g^{-1}h^{-1}) \qquad [3.23]$$

where Q_{O2} and Q_{CO2} are the oxygen uptake rate and CO_2 production rate measured on line using UBICON (see equations 3.5, 3.6)

The biomass yield on consumed sugar ($Y_{X/S}$)and on oxygen ($Y_{X/O2}$) were calculated as follows:

$$Y_{X/S} = \frac{\mu}{q_S} \qquad (g\ g^{-1}) \qquad [3.24]$$

$$Y_{X/O} = \frac{\mu}{q_{O_2}} \qquad (g\ mmol^{-1}\ \text{ or }\ g\ g^{-1}) \qquad [3.25]$$

For the alginate yield on consumed sucrose ($Y_{alg/S}$) the following formula was used

$$Y_{alg/S} = \frac{q_{alg}}{q_S} \qquad (g\ g^{-1}\ ) \qquad [3.26]$$

4. RESULT AND DISCUSSION

4.1. Role of dissolved O_2 concentration on cell growth and alginate production in pO_2 controlled bioreactor

Although *azotobacters* are known to be extremely sensitive to oxygen when grown diazotrophically quantitative data concerning the effect of dissolved oxygen tension on alginate production are still needed. Many authors used during their studies either shaked flasks (Horan et al. 1981; Okabe et al. 1981; Chen et al. 1985; Brivonese and Sutherland, 1989; Savalgi and Savalgi, 1992; Clementi et al. 1995; Pena et al. 1997) or fermentors without accurate control of pO_2 (Jarman et al, 1978; Annison and Couperwhite, 1986 a, b; Parenete et al. 1998). The influence of pO_2 values on alginate production is still not tested for the strain DSM 93-541b and hence, the first aim of this study is to determine the pO_2 spectra for the strain used under fixed agitation speed.

In the first four experiments, different dissolved oxygen concentrations were tested in order to determine their effect on cell growth, PHB production as well as alginate production by diazotrophically growing cells. pO_2 was controlled in the range of 1-10% air saturation by mixing pure oxygen and nitrogen in the inlet gas and controlled by UBICON. To exclude possible effect of phosphate limitation, phosphate mixture was added in excess to the salt medium (800mg/l in the ratio of 1 KH_2PO_4: 4 K_2HPO_4) and exhaustion of phosphate was never observed. The results of these experiments are graphically represented in **Figs. 4.1-4.4.**

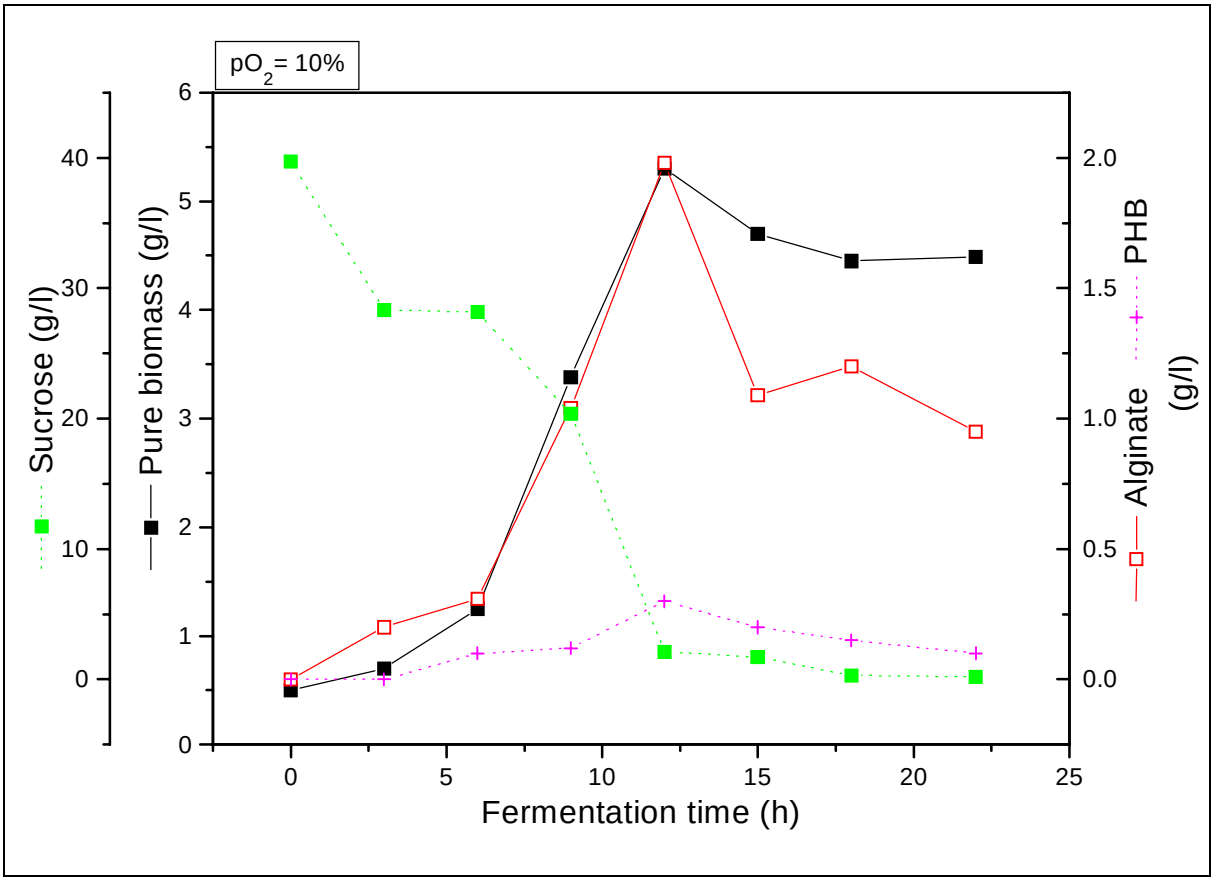


Fig. 4.1 Batch fermentation with a constant pO_2 of 10% air saturation.

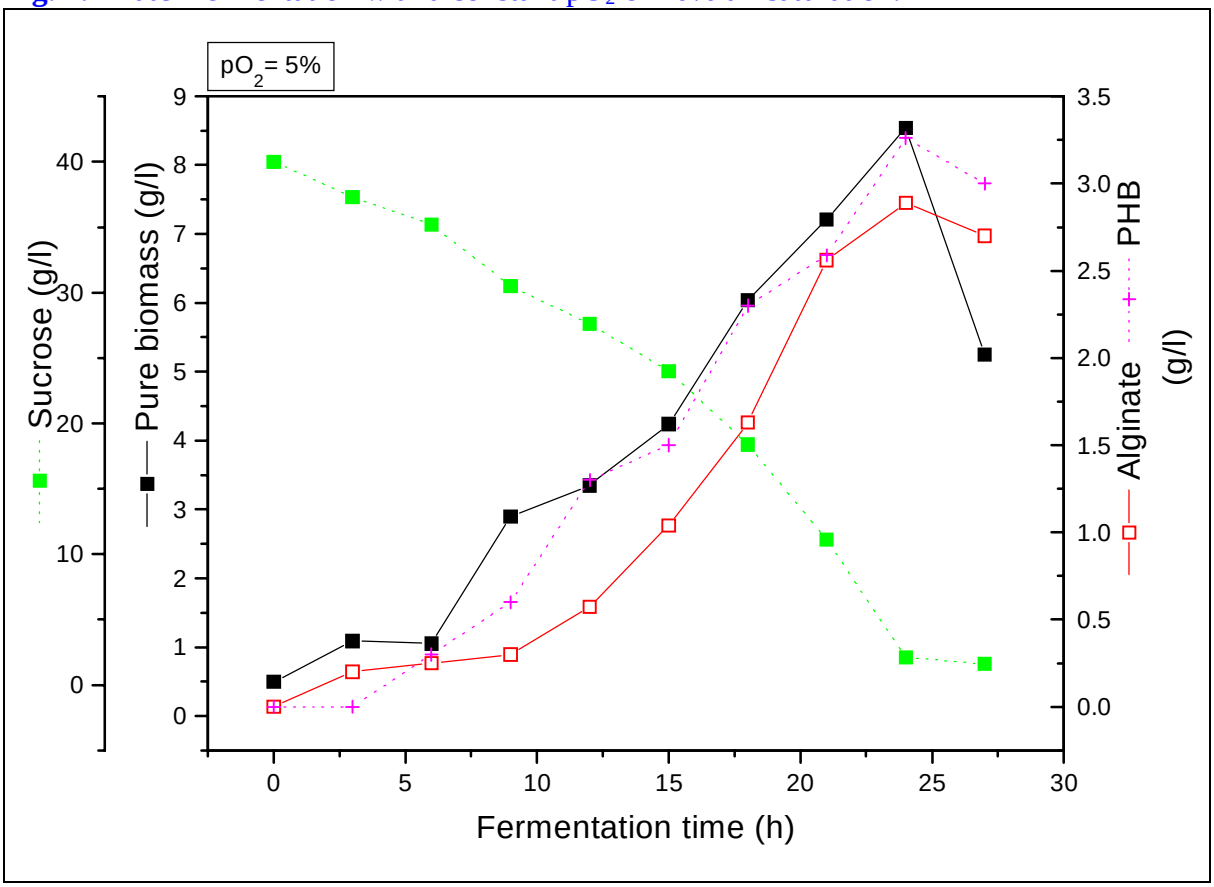


Fig. 4.2 Batch fermentation with a constant pO_2 of 5% air saturation.

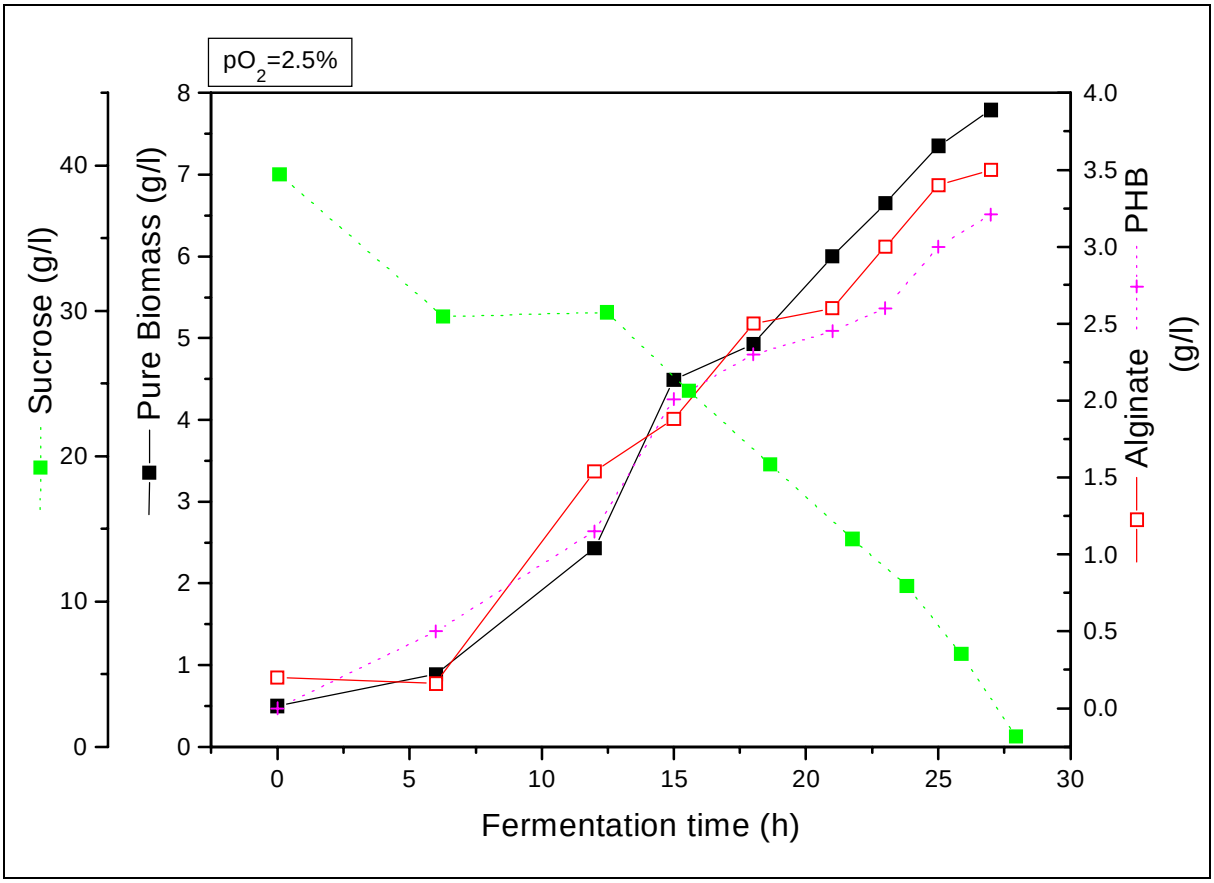


Fig. 4.3 Batch fermentation with pO_2 of 2.5 % air saturation.

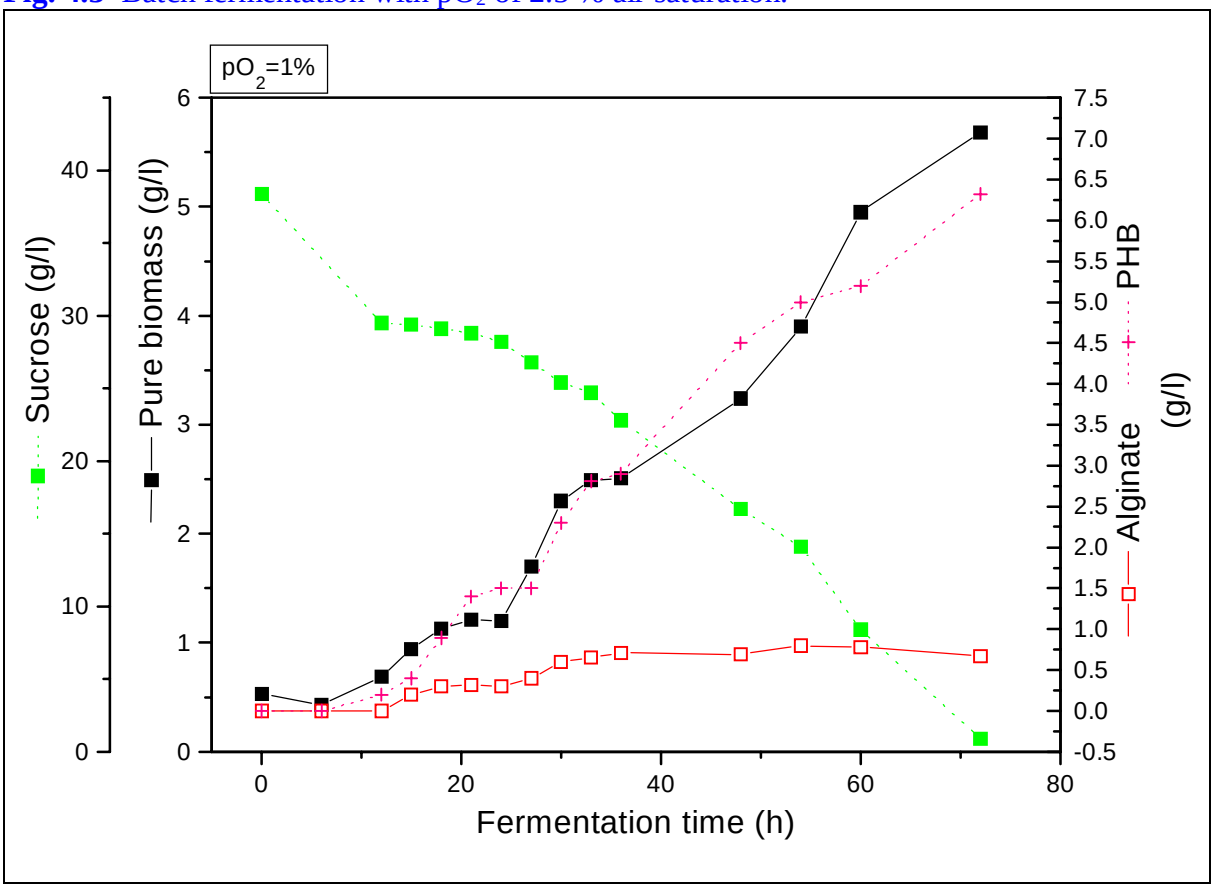


Fig. 4.4 Batch fermentation with pO_2 of 1 % air saturation.

4.1.1. Specific growth rate as affected by different pO_2 values

It is clear from the data (shown in **Tab. 4.1**) that the extent of logarithmic growth was approximately proportional to the increase in dissolved oxygen tensions up to 5% air saturation. Maximum growth rate of 0.236 h^{-1} with mean generation time of 6.2h was obtained at the highest pO_2 value (10%) compared to μ_{max} of 0.1 h^{-1} with mean generation time of 19.8h at very low dissolved oxygen tension. However, from plotting the biomass logarithm, the lag periods were not observed at pO_2 of 1, 2.5 or 5% and a very short lag period of 1.3h was observed at the highest pO_2 value (10%).

The basic causes of lag in growth are either change in nutrition or physical environment, presence of inhibitors or the physiological state of the inoculum culture (Pirt, 1985). Since in the four experiments the age of the inoculum culture was the same (mid logarithmic), the low aeration condition and probably the lower pO_2 value in the shaked inoculum culture is responsible for the lack of such a period under lower pO_2 values (1-5%). However, the short lag period (1.3h) at higher pO_2 value (10%) may be an expression of the time required to overcome the inhibitory effect of oxygen through respiratory protection.
Although growth was faster when pO_2 was increased, the maximum biomass concentration was found at pO_2 of 5% air saturation as can be seen from the biomass yield (**Tab. 4.1**). This is in agreement with results obtained by Parente et al. (1998) using the same bacterium (*A. vinelandii* DSM 576) at different pO_2 values.

Tab. 4.1 The effect of different pO_2 values on the length of lag period, growth yield, μ ,μ_{max} and the mean doubling time of *A. vinelandii* grown diazotrophically.

pO_2 (%)	Lag period length (h)	Y_{XS} (g/g)	μ^* (h^{-1})	Mean Doubling time (h)	μ_{max}^* (h^{-1})
1	-	0.163	0.035	19.8	0.1
2.5	-	0.289	0.106	6,5	0.18
5	-	0.305	0.116	5,9	0.18
10	1.3	0.172	0.111	6,2	0.236

*calculated from biomass without PHB.

4.1.2. Alginate and PHB biosynthesis

Generally, alginate production was parallel to biomass production in almost all fermentations (except for oxygen-limited condition, at pO_2 value of 1%, where another product namely PHB accumulated) and ceased with the exhaustion of sucrose in the medium.

At the optimum pO_2 value for alginate production (2.5%), the carbon source was mainly directed to alginate biosynthesis as indicated by the highest alginate yield ($Y_{alg/X}$) of 0.45g/g (compared to 0.33 and 0.21g/g at pO_2 values of 5 and 10% air saturation, respectively). On the other hand, lower pO_2 values (1%) resulted in a very low alginate yield (0.11g/g) and the carbon source was mainly directed to PHB biosynthesis (comprising 44-58% of the biomass).

Increasing the pO_2 values resulted in decreased PHB yield ($Y_{PHB/X}$) form 1.4, 0.5, 0.4 to 0.04 g/g at 1, 2.5, 5 and 10% air saturation, respectively (**Fig. 4.5**). These results were in agreement with those obtained by Page et al. (1997) and Bormann et al. (1998) using different *Azotobacter* spp.

As depicted in **Fig 4.6** and in terms of alginate productivity, alginate concentration and alginate yield, pO_2 values between 2.5-5% air saturation were found to be optimal for alginate production by this bacterium. Above and below this pO_2 range, the strain either wasted the carbon as PHB or as CO_2. It was also noted that the fermentation time decreased sharply with the increase in pO_2 which resulted in elevated alginate productivity at higher pO_2 levels. Values between 2-5% of air saturation were also reported to support the maximum rate of alginate biosynthesis by diazotrophically growing *Azotobacter* cells (Horan et al. 1983). However, using a complex nitrogenous medium, Parenta et al. (1998) reported that batch fermentation with uncontrolled pO_2 gave a higher alginate concentration and yield than batch fermentation with controlled pO_2 at 5 and 2% air saturation.

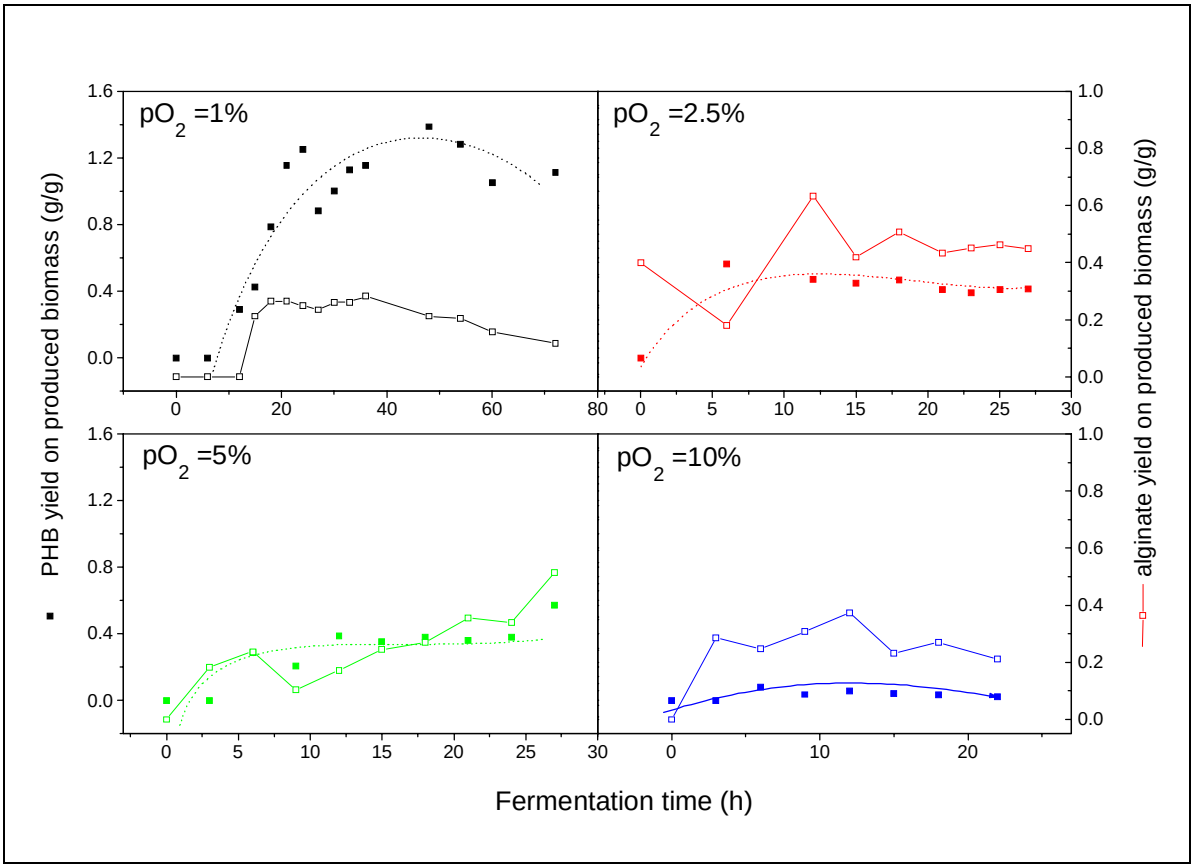


Fig. 4.5 The effect of different pO_2 values on PHB and alginate yield by *Azotobacter vinelandii* grown diazotrophically.

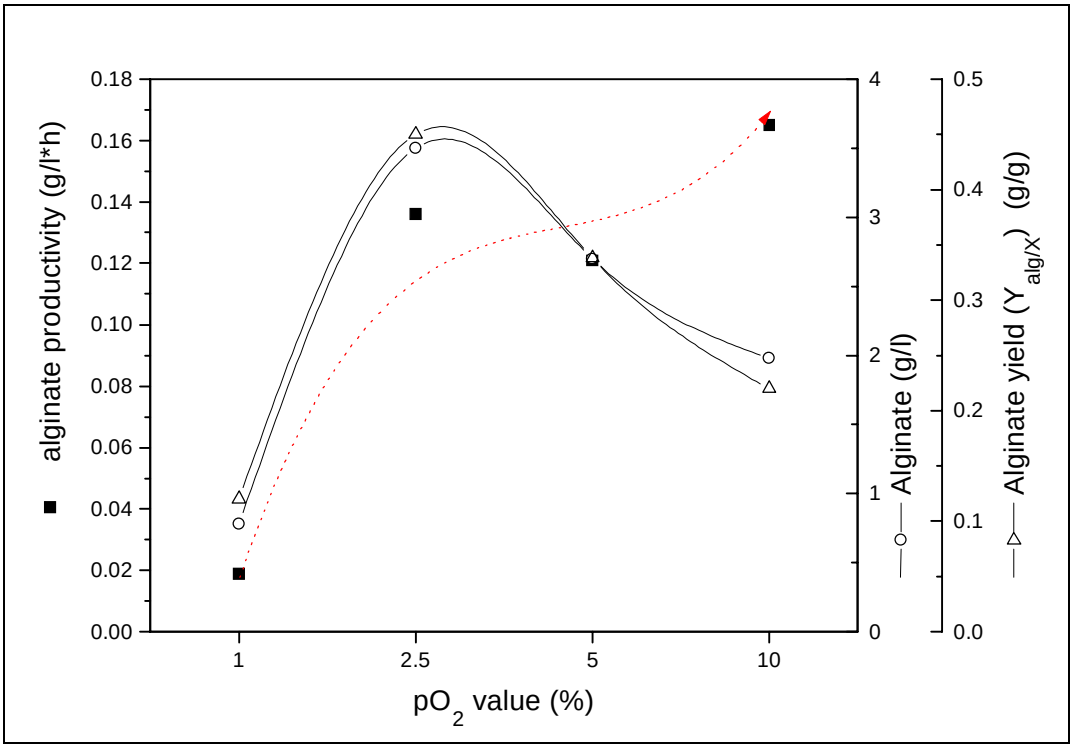


Fig. 4.6 Volumetric alginate productivity, alginate concentration and alginate yield as affected by different dissolved oxygen tension values.

4.1.3. Specific sucrose consumption rate as affected by different pO_2 values

The results obtained in **Fig. 4.7** strongly suggest that the specific rate of sucrose consumption (q_s) increased in direct proportion to the increase in dissolved oxygen tension. A mean qs value of 0.2, 0.5, 0.6 and 1.4 g/g*h were calculated at 1, 2.5, 5 and 10% pO_2, respectively.

Since diazotrophically growing cells of *Azotobacter vinelandii* are known to be sensitive to oxygen, the increased q_s values could be attributed to the increase in respiratory protection with increasing pO_2 values. By catabolising the available carbon source, the cells remove oxygen which is an inhibitor of the highly reduced nitrogenase system.

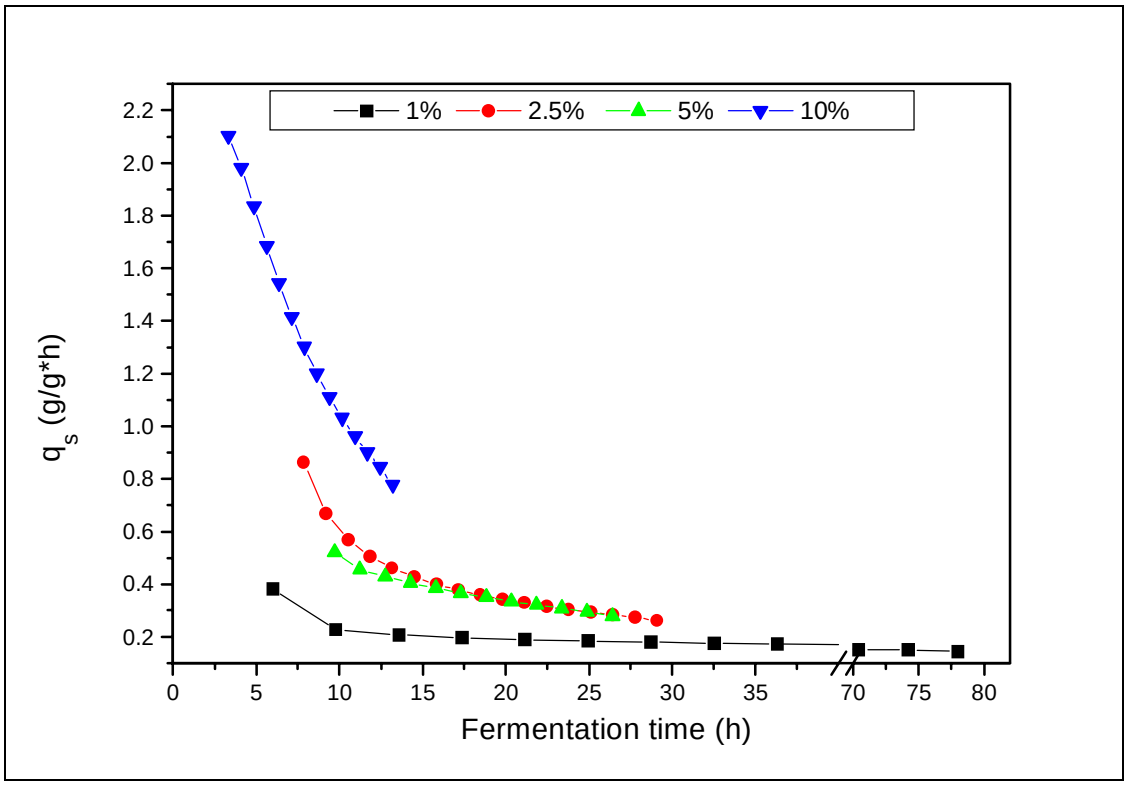


Fig. 4.7 q_s as a function of different dissolved oxygen tension.

4.1.4. Summary

In view of these facts, dissolved oxygen concentration has a major effect on the carbon and energy metabolism, growth rate and fates of the carbon source (shift of metabolism). A low pO_2 value (1% air saturation) activates the accumulation of PHB as the main product, whereas alginate biosynthesis is favoured by intermediate pO_2 values (2.5 –5%). At very high

dissolved oxygen tension, very small amount of PHB was formed, the growth rate was maximum and most of the carbon was wasted in respiration.

4.2. The effect of different nitrogenous rich compounds on bacterial growth and alginate yield in shake flask culture

In order to lower the extreme sensitivity of the diazotrophically growing bacterium to oxygen, different nitrogenous rich compounds were added to the medium.

Fig. 4.8 demonstrates that the incorporation of nitrogenous compounds into the medium did not increase the alginate yield by this strain. Although alginate productivity (g/l*h) recorded the highest value when either Bacto peptone or protease peptone were added, the highest alginate yield (g alginate/g biomass) was from cells grown diazotrophically. It was also noticed that alginate yield as well as productivity were lowest when ammonium nitrate was the incorporated nitrogen source.

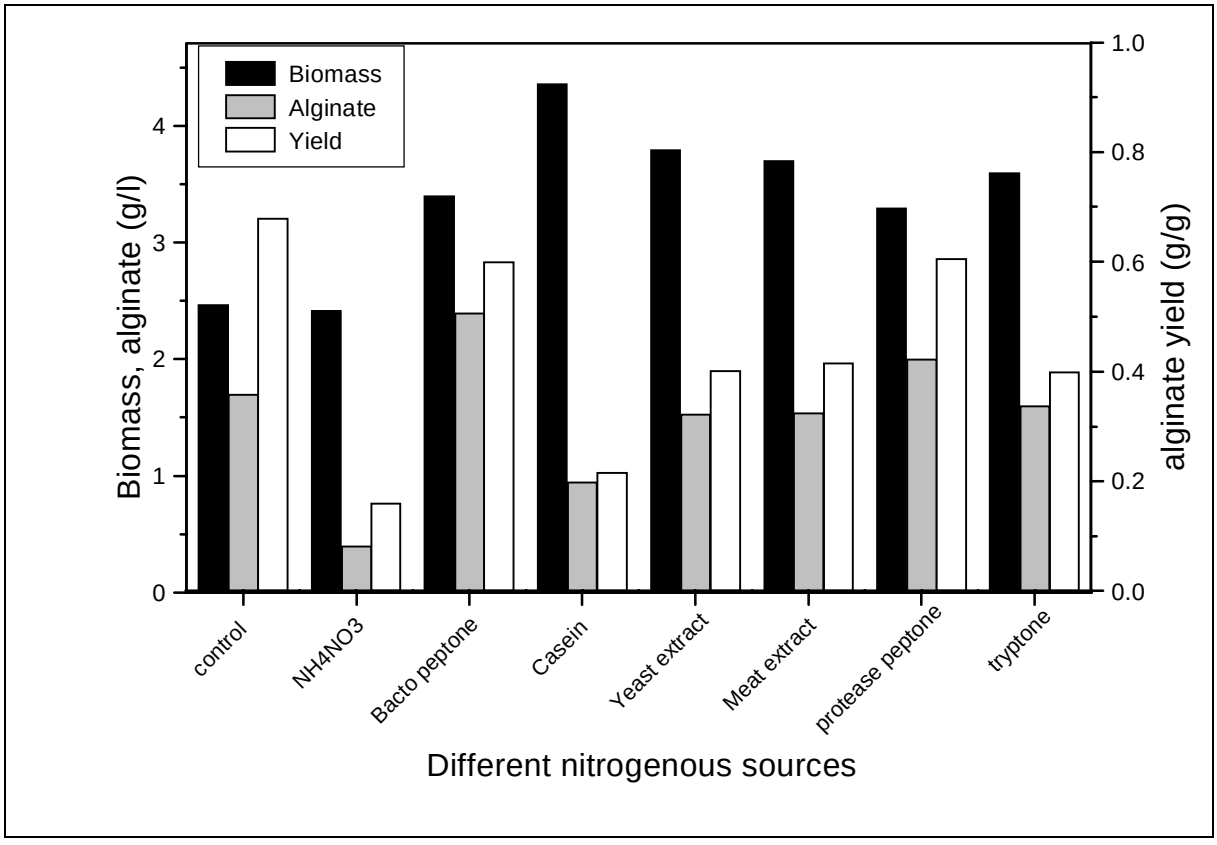


Fig. 4.8 The effect of different nitrogenous rich compounds on biomass production and alginate yield.

The addition of nitrogenous sources (except ammonium nitrate) had a positive effect on PHB production as it peaked in the presence of meat extract and 4.5g was accumulated in comparison to 0.5g at diazotrophically growing cells (**Fig. 4.9**). Nitrogen fixation and respiration should conflict with PHB production since all three reactions are sinks for reducing power. Thus N_2- fixing cells have a very low PHB content and the addition of organic nitrogen spares the need for nitrogen fixation and the respiratory protection of the oxygen labile nitrogenase complex and thus allows the reducing power and Acetyl CoA derived from active sugar metabolism to be used for PHB production (Page et al, 1997). Similar result were obtained recently by Pal et al. 1998 using an *A. chroococcum* strain.

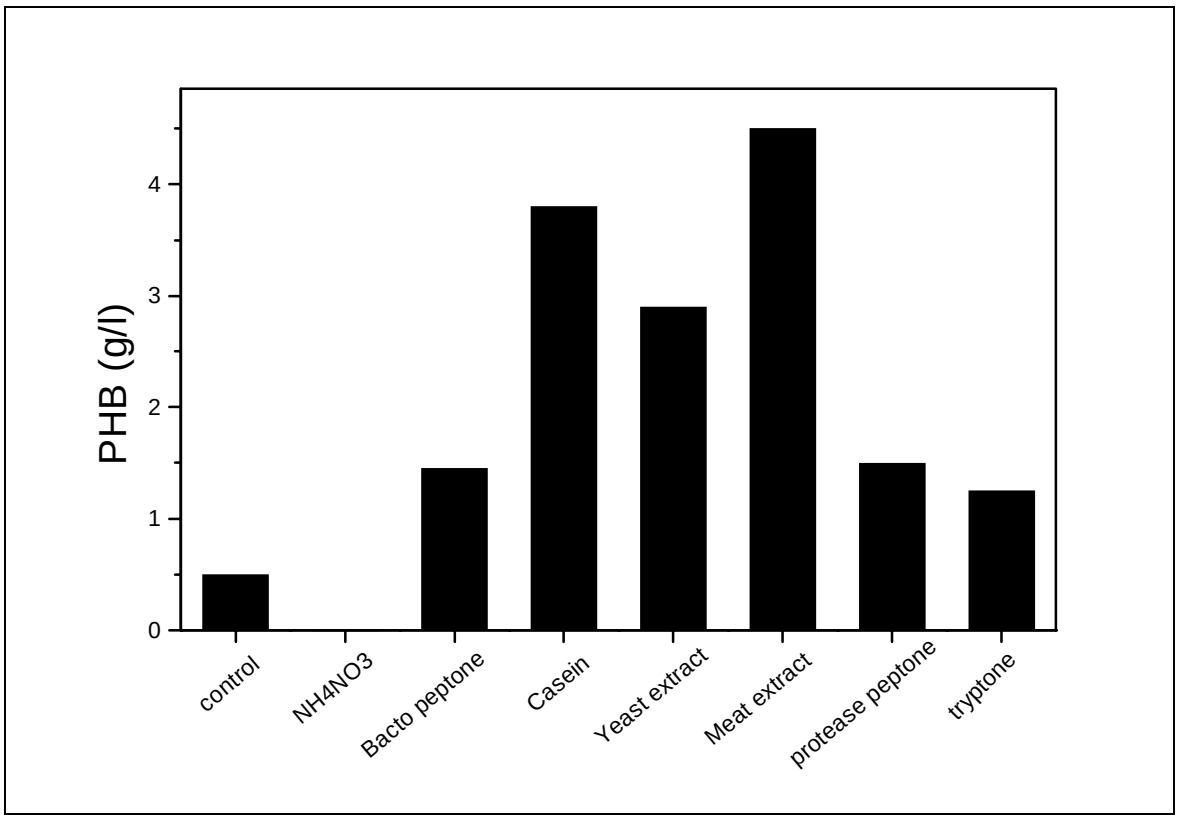


Fig. 4.9. PHB production as a function of different nitrogenous sources.

To eliminate the effect of oxygen limitation, the effect of different nitrogenous compounds on alginate, biomass and PHB production were also tested at different agitation speeds (**Fig. 4.10**).

It was noted that the different nitrogen sources gave better cell yields than the nitrogen free medium while alginate yield (g/g biomass) was higher in the nitrogen free medium. It was also

noticed that the bacterium exhibits oxygen sensitivity even in the presence of nitrogenous compounds as was noticed by the lower cell yield at higher agitation speeds.

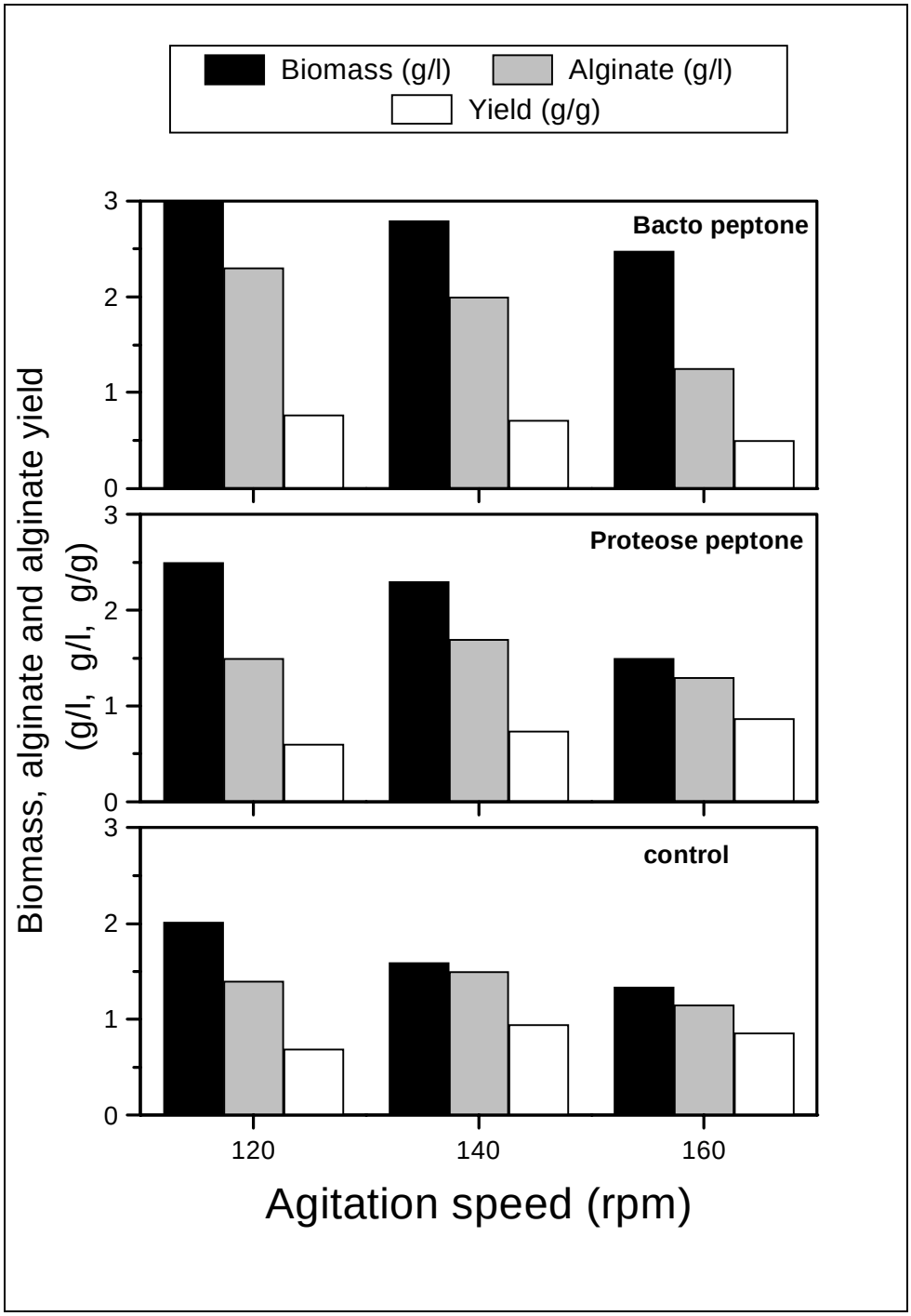


Fig. 4.10 The effect of aeration rate and different nitrogenous compounds on biomass, alginate and product yield by *Azotobacter vinelandii* grown in shaked flasks.

Based on the experimental data it seems reasonable to say that the addition of complex nitrogenous compounds to the growth medium of *A. vinelandii* had no significant advantage for the production of alginate because of the following reasons.

- a significantly higher alginate yield (g/g) was obtained in nitrogen free medium compared to that in complex medium.
- the cells exhibit oxygen sensitivity even in the presence of complex nitrogenous materials.
- the production of PHB by this bacterium was markedly favoured by the addition of complex nitrogenous materials.

and

- for medium simplicity and for further calculations

4.3. Effect of different phosphate levels on alginate production in shake flasks as well as in bioreactor

In the previous experiments, to study the effect of dissolved oxygen tension an alginate, PHB and biomass production, the phosphate mixture was added in excess (800mg/l). However, excess phosphate resulted in higher biomass and consequently a higher respiration rate. And since only pure oxygen was necessary to reach and control the desired pO_2 value during the fermentation runs, it is reasonable from the commercial point of view to reduce the overall oxygen consumption and to use air instead of pure oxygen by lowering the phosphate concentration. Furthermore, in literature both phosphate-rich and phosphate-limited conditions have been reported to be advantageous for alginate production in *A. vinelandii* (Okabe et al. 1981; Chen et al. 1985; Savalgi and Savalgi. 1992; Clementi et al. 1995; Parenta et al. 1998). Thus the aim of the following experiments is to compare the effect of different phosphate concentrations in shake flasks (pO_2-uncontrolled system) and in bioreactor (pO_2-controlled system) so as to explain the inconsistency data reported in the literature.

4.3.1. Different phosphate level in uncontrolled-pO_2 system

The effect of different phosphate levels on alginate production by *Azotobacter vinelandii* growing diazotrophically was studied first in shaked flask cultures. The results represented in **Fig. 4.11** and **4.12** indicate that increasing the phosphate level (from 80mg to 800mg/l) increased the cell density as well as the alginate concentrations. However, alginate yield recorded its maximum at the lowest phosphate concentration.

An inspection of the data shown in **Fig. 4.12** revealed that the growth rates of the nitrogen fixing bacterium are strongly dependant on the initial phosphate concentration and reached their maximum at the highest concentration. Surprisingly, the phosphate mixture was not totally consumed in the culture medium, even at the lowest concentration (**Fig. 4.11**). A difference in $q_{phosphate}$ with different initial phosphate concentrations and consequently different values of K_s may explain these results.

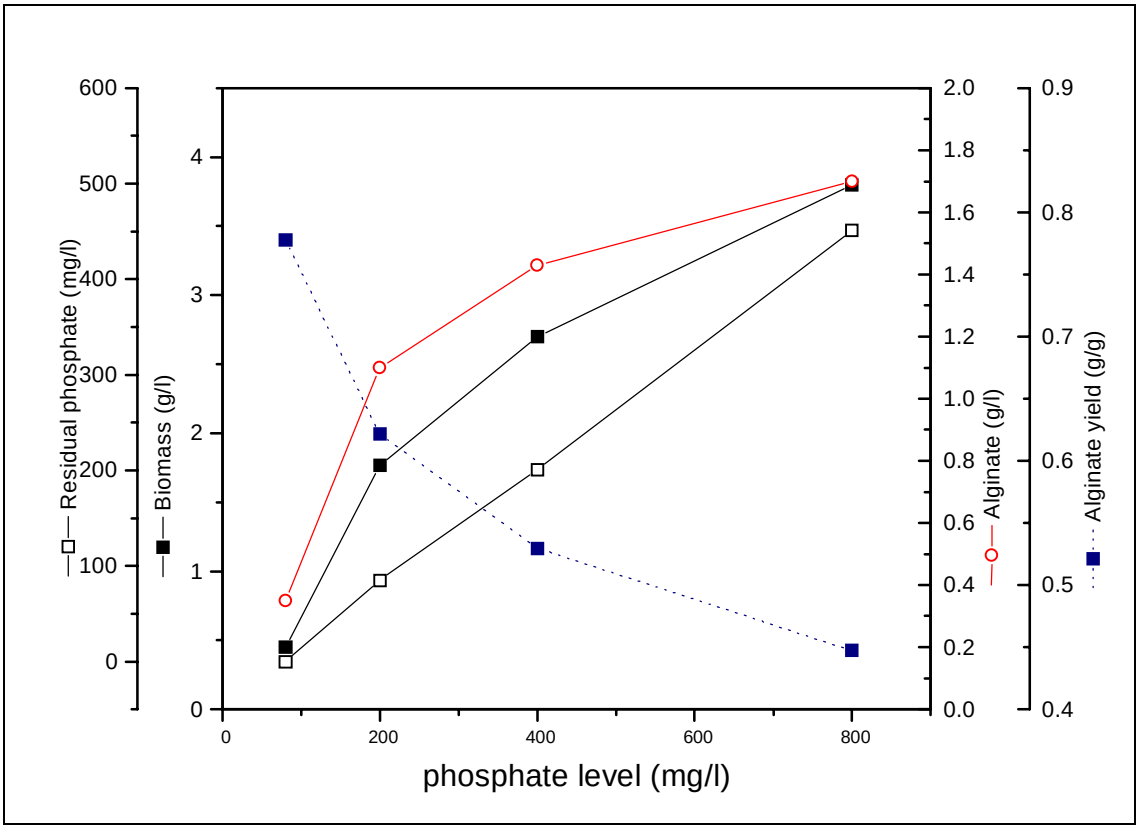


Fig. 4.11 The effect of different phosphate levels on biomass formation, alginate production as well as alginate yield of nitrogen fixing *Azotobacter vinelandii*.

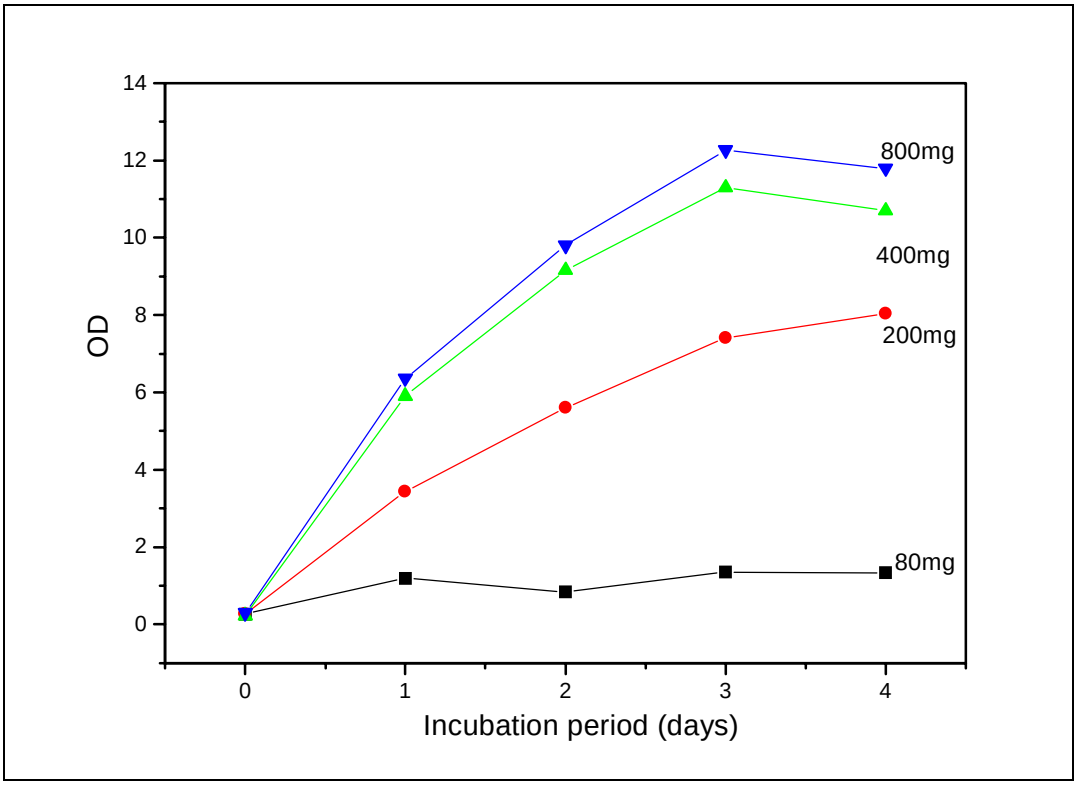


Fig. 4.12 The growth behaviour of *Azotobacter vinelandii* at different initial phosphate concentrations in shaked flasks.

It is worth noting that in this experiment an important factor, the dissolved oxygen tension, was not incorporated and the peak oxygen demand during the late exponential phase of growth coupled with the effect of viscosity on aeration of the culture, is likely to induce oxygen limitation.

In view of the experimental data demonstrated in section 4.1, the control of dissolved oxygen tension was found to be important for the shift in metabolism, growth rate and alginate production, the effect of phosphate limitation on alginate production by this strain cannot be properly investigated in shake flask experiments since the dissolved oxygen tension is not controlled.

For example, if the pO_2 in the shacked flasks was high (at the beginning of the fermentation), excess phosphate would be required for respiration to protect the nitrogenase system from being damaged with molecular oxygen (respiratory protection) (**Fig. 4.13**). This, however, explains the behaviour of the biomass formation with increasing phosphate concentration in the medium and the failure of growth at very low phosphate concentrations.

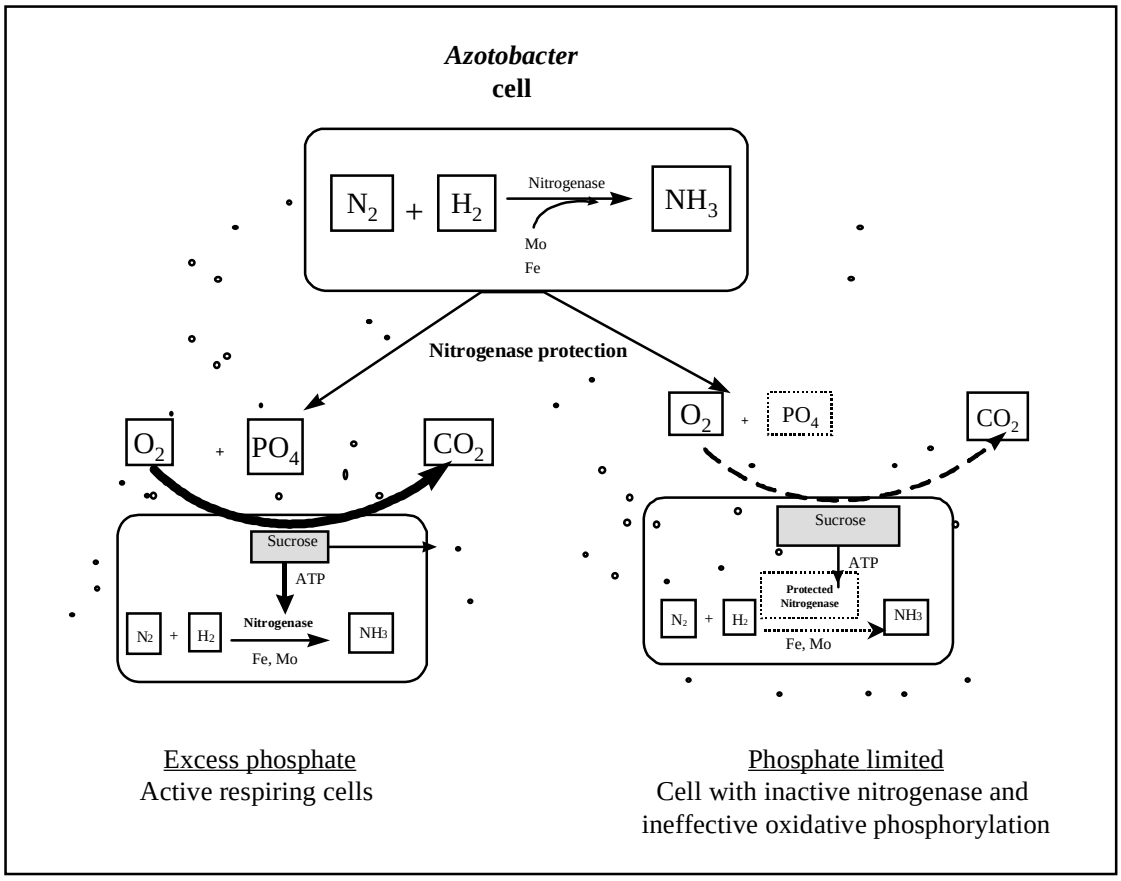


Fig. 4.13 The respiratory protection of *Azotobacter vinelandii*.

Phosphate limited culture of *Azotobacter vinelandii* is known for its extreme sensitivity to molecular oxygen. Tsai et al. (1979) concluded that phosphate limited cultures stop growing in the presence of oxygen because ADP is converted to ATP and the resulting increased ratio of ATP to ADP shuts off respiration. Thus, the aim of the following experiment was to study the effect of phosphate limitation in pO_2 controlled bioreactor.

4.3.2. Effect of different phosphate levels on cell growth and alginate production in pO_2 controlled bioreactor

The effect of different phosphate levels on alginate production was evaluated in the following 3 fermentation runs with 400, 200 and 100mg/l phosphate. The pO_2 was kept constant in these experiments at the optimum value for alginate production (2.5-3% air saturation).
The results of these runs are presented in **Figs. 4.14 - 4.16.**

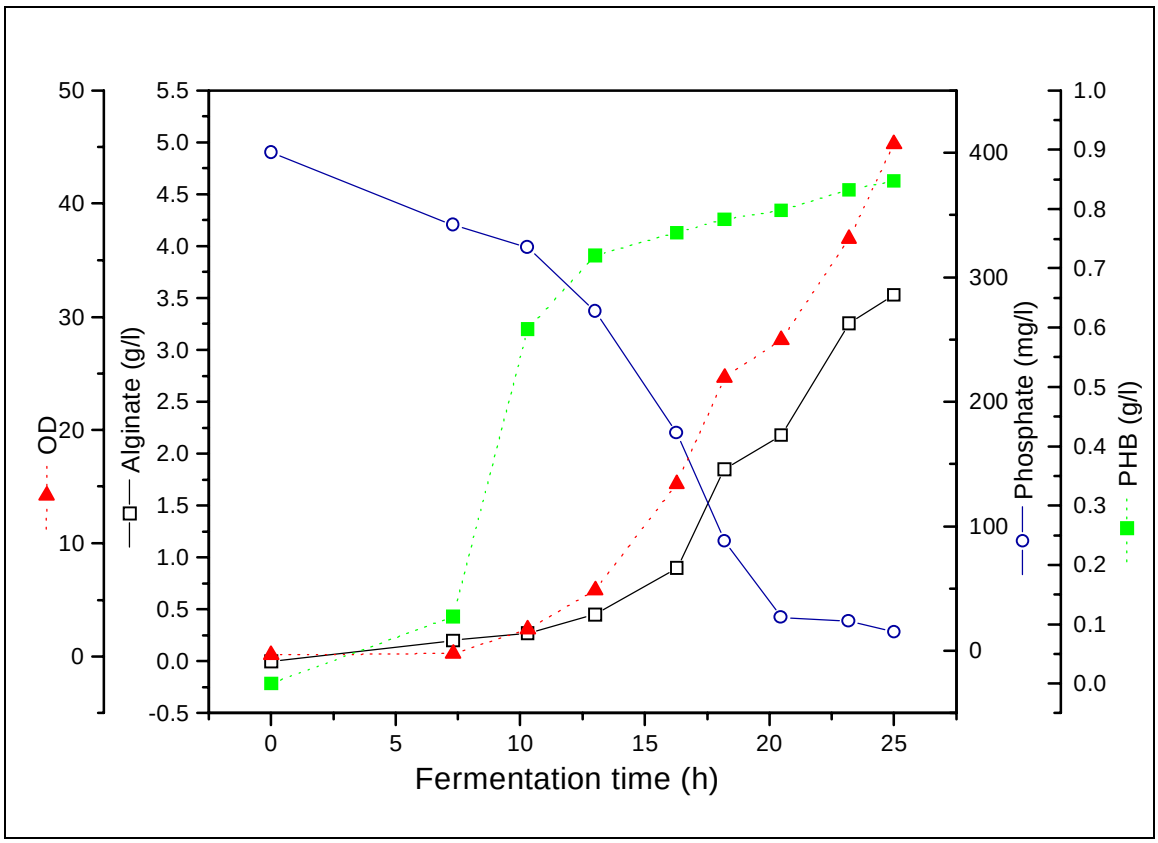


Fig. 4.14 Time course of batch fermentation with phosphate concentration of 400mg/l.

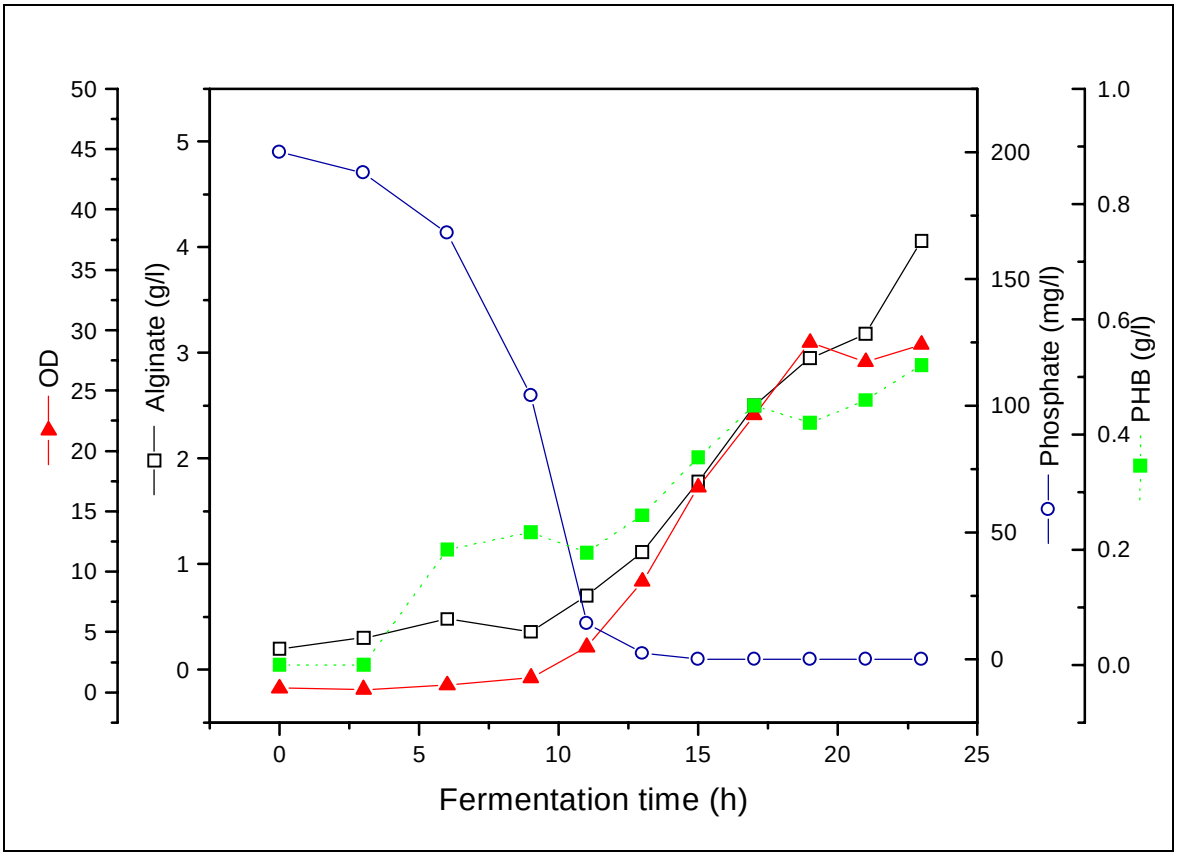


Fig. 4.15 Performance of batch process of *Azotobacter vinelandii* with 200 mg /l phosphate.

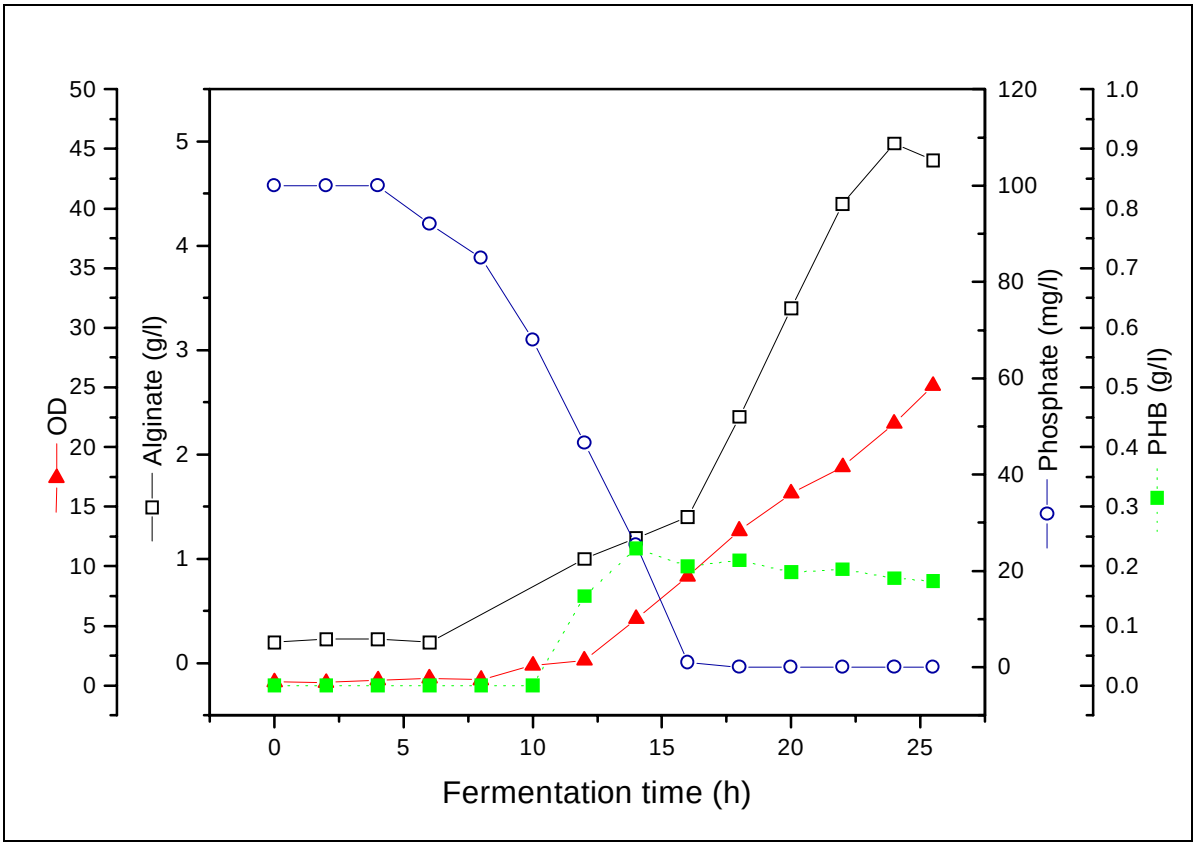


Fig. 4.16 Time course of fermentation run with low phosphate concentration (100mg/l).

4.3.2.1. Biomass formation

The evaluation of biomass during the time course of the fermentation under different phosphate levels was compared in **Fig. 4.17**.

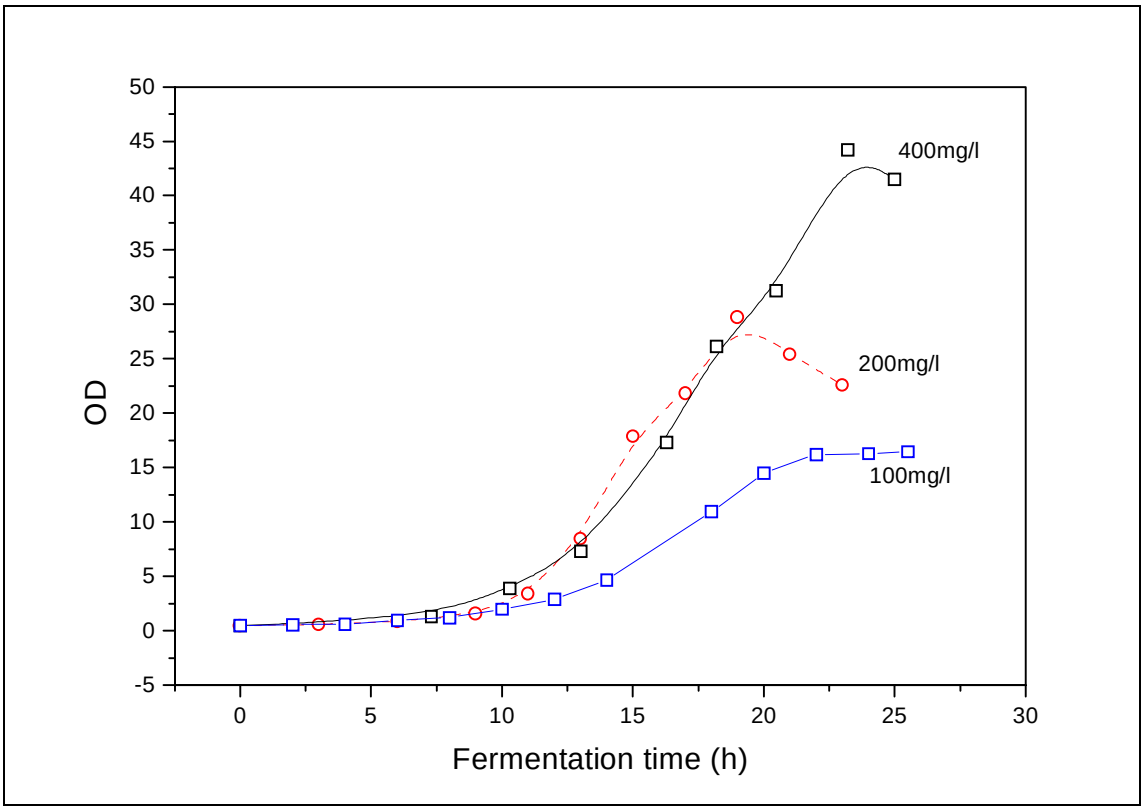


Fig. 4.17 Time course of microbial growth under different phosphate levels.

As phosphate concentration increased, the level of biomass also increased, with the biomass yield being highest at a high phosphate level (0.37g/g at 400mg/l), intermediate (0.24g/g at 200mg/l phosphate), and lowest (0.21g/g at 100mg/l phosphate). This was in agreement with the results of the shacked flask experiment (**Fig. 4.12**).

4.3.2.2. Alginate production

On the contrary, the alginate production increased significantly when the initial phosphate concentration decreased (**Fig. 4.18**) and the best phosphate concentration for alginate production was found to be the lowest concentration (100mg/l), which gave 5g/l compared to 4.01g/l and 3.5 g/l for 200mg/l and 400mg/l phosphate content, respectively.

Alginate yield (on biomass formed $Y_{(p/x)}$) as well as alginate productivity recorded the highest value (0.67g/g and 0.21g/l*h) at lowest phosphate level (100mg/l) compared to 0.27g/g and 0.14g/l*h at highest phosphate concentration (400mg/l, respectively).

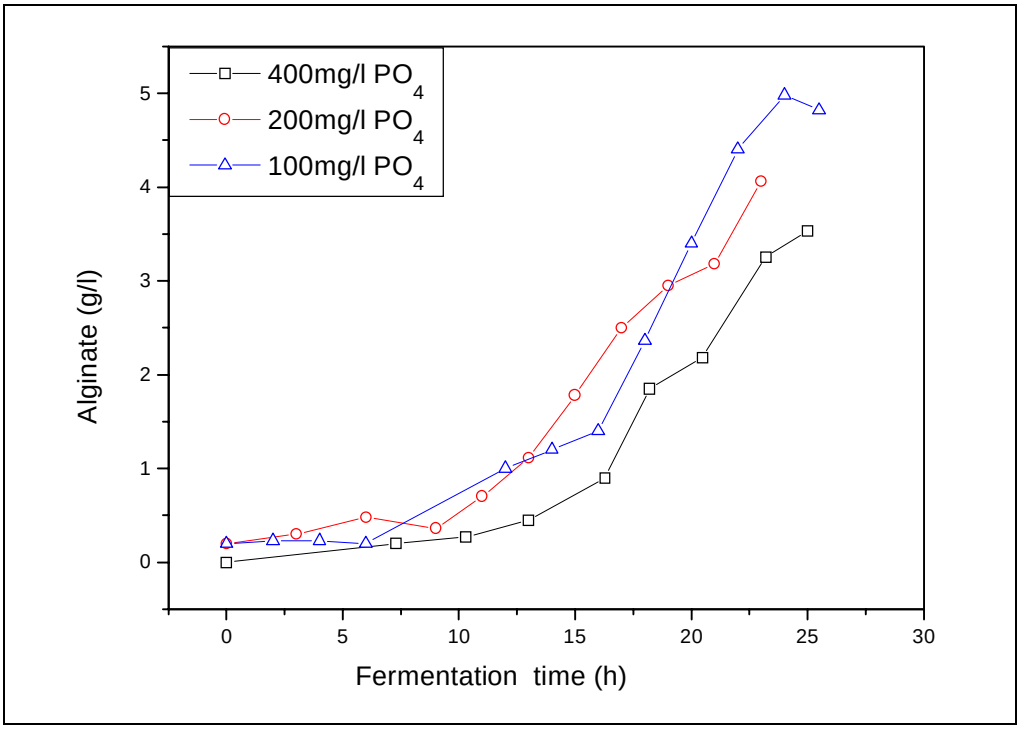


Fig. 4.18 Comparative study of time course of alginate production at different phosphate concentrations.

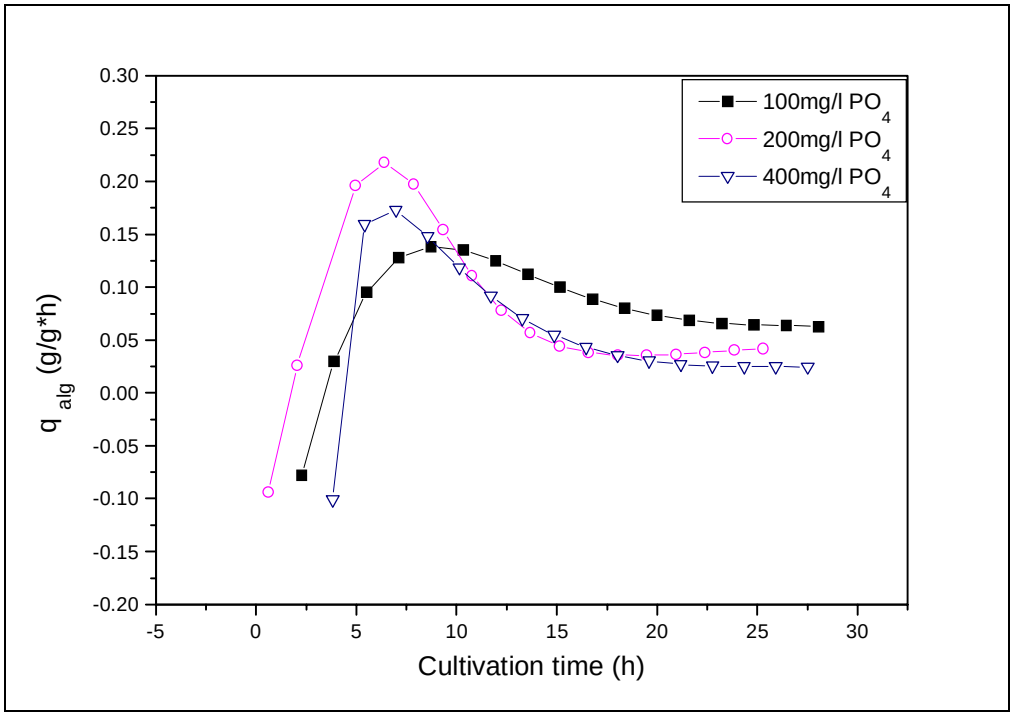


Fig. 4.19 Specific alginate formation rate as influenced by different phosphate concentrations.

From **Fig.** 4.19, it was noted that the specific alginate production rate (q_{alg}), which was highest (0.06 g/g*h) at the lowest phosphate level (100mg/l), peaked at few hours after the beginning of fermentation then decreased gradually with time. This may be due to the increase in the effective viscosity of the pseudoplastic culture media with time or/and inadequate mixing and presence of oxygen limitation at some points. Similar behaviour was also observed in xanthan fermentation by *Xanthomonas compestris* (Peters et al. 1989).

It should be stressed that, most of the alginate was produced in the phosphate-limited growth phase of these cultures. It is worth mentioning that these results differed considerably from those obtained from shake flasks without pO_2 control since in shake flasks, the best alginate production was obtained at the highest phosphate concentration (800mg/l) tested. These pronounced differences may be due to the heterogeneous pO_2 profile during the fermentation run in flasks. Furthermore, slow growth was observed at an initial phosphate concentration of 80 mg/l in shake flask culture although the strain can grow even at lower concentrations of phosphate in a controlled microaerophilic bioreactor (pO_2 of 2-5% air saturation). This may be due to a too high pO_2 at the beginning and an ineffective oxidative phosphorylation under these conditions (Haldenwang and Behrens, 1983).

4.3.2.3. PHB biosynthesis

The behaviour of PHB formation differed significantly from that of alginate production. As the phosphate level increased in the culture medium the PHB content in the biomass increased and reached its maximum when phosphate level was highest (400mg/l). **Fig. 4.20** shows the time course of PHB formation in the three runs with different phosphate levels.

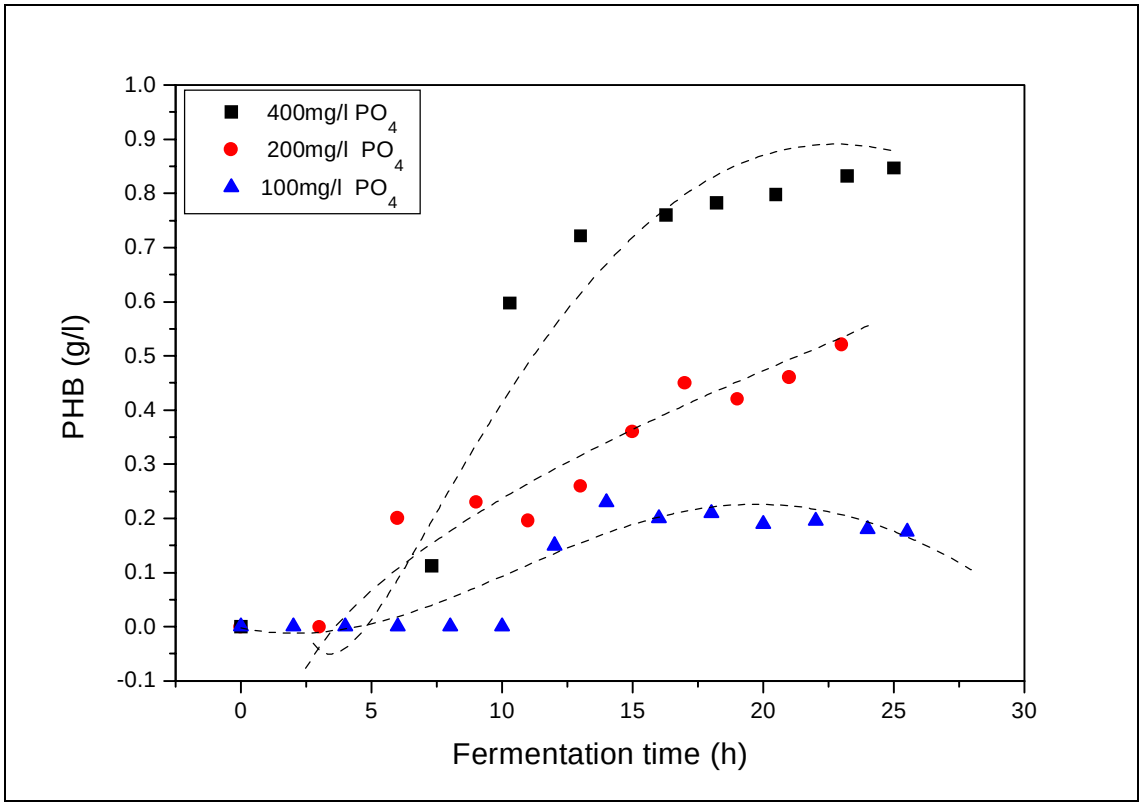


Fig. 4.20 PHB production as a function of different initial phosphate concentrations.

4.3.2.4. Molecular weight and G/M ratio

Fig. 4.21 shows the effect of different phosphate concentrations on polymer quality in terms of relative molecular weight and G/M ratio. Generally, the mannuronic acid content of alginate decreased during the fermentation course. It is well known that alginate is first produced as pure mannuronic acid, then alginate epimerase is secreted to epimerise some mannuronic acid monomers to guluronate. This explains the increase in guluronic acid residues of the alginate samples with time.

The molecular weight, on the other hand, was relatively stable during fermentation time till a phosphate limited phase was reached, followed by a decrease in molecular weight. This may be due to the secretion of alginate lyase from the cells.

Comparing the three different phosphate runs, the relative increase in the molecular weight of the alginate samples was always accompanied by an increase in the mannuronic acid content.

According to the theory of Gacesa (1987), both alginate lyase and alginate epimerase show similarities in their reaction mechanisms and these allow the conversion of lyases to epimerases and vice versa, i.e higher molecular weight should be accompanied with high mannuronic acid content. This theory, in fact, may explain the results obtained by this experiment.

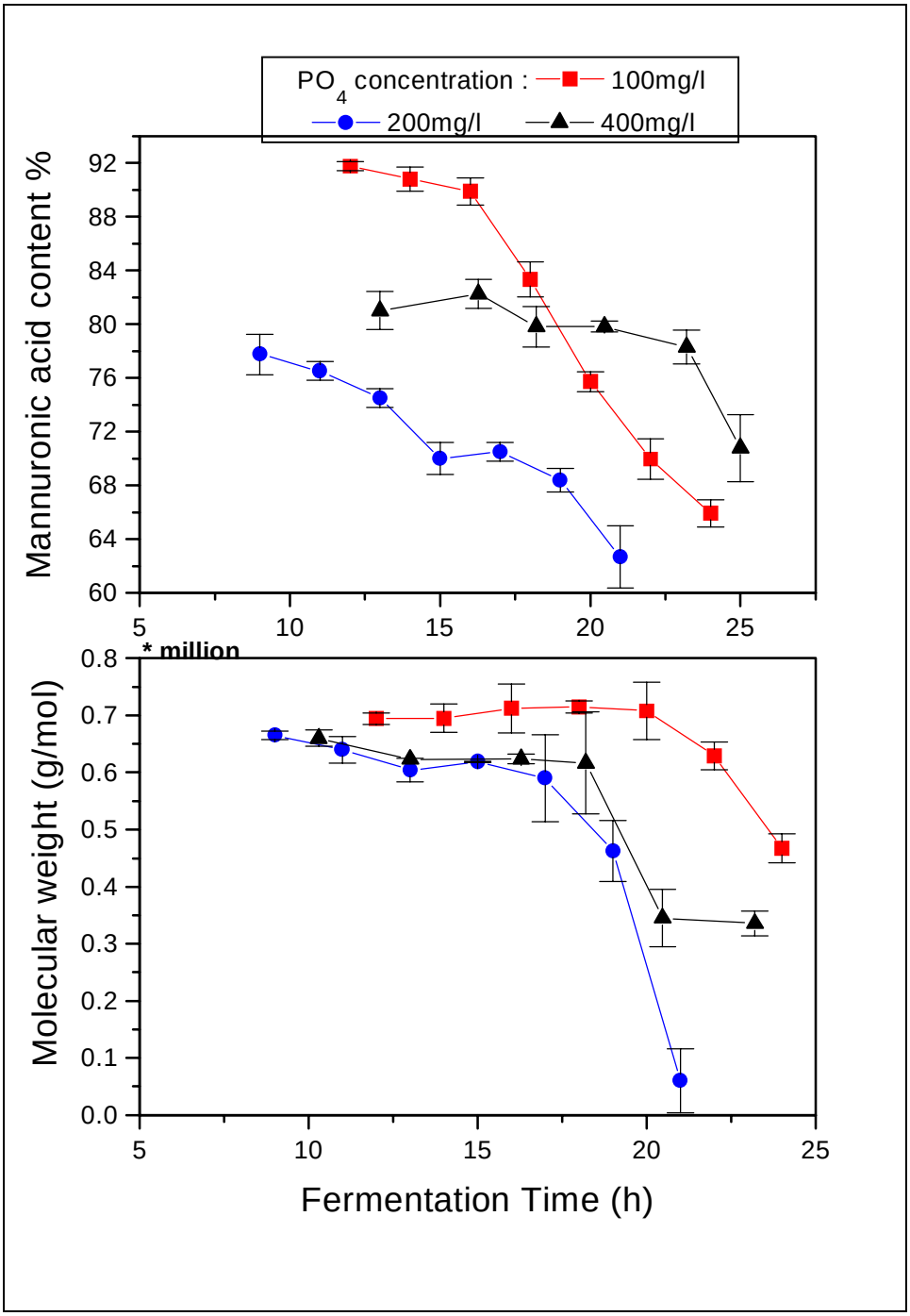


Fig. 4.21 Relative molecular weight and mannuronic acid content as a function of different phosphate levels.

Since the volumetric mass transfer coefficient for oxygen K_La decreases with increasing viscosity, the high molecular weight of alginate samples at the lower phosphate levels is advantageous for the oxygen sensitive cells. In addition a slime covering (capsule) would impede access of oxygen to sensitive sites and might thus have a survival value.

4.3.2.5. Phosphate rich - phosphate limited culture and optimum RQ for alginate production

The results presented in **Figs. 4.14**, **4.15** and **4.16** proved that alginate was produced in both growth conditions, i.e. phosphate rich and limited cultures. However, most of the alginate was produced in the phosphate limited phase of growth.

Fig. 4.22 shows the significant differences in the RQ profile recorded on-line in the three fermentation runs with different phosphate concentrations. In general, RQ was lower in cultures with a lower initial phosphate content. This is particularly obvious during the phosphate-limited period of cultivation. At the onset of phosphate limitation, a sudden increase of RQ was observed. The unusual high RQ values calculated under these conditions were apparently caused by the dynamics of mass transfer of O_2 and CO_2 under conditions of unbalanced consumption and mass transfer. In fact, the pO_2 values of the cultures also quickly jumped due to an abrupt decrease in oxygen uptake rate (data not shown). Then the cultures gradually adapted to phosphate limitation. Under these adapted conditions, average RQ values of 1.46, 1.30, and 0.89 were calculated for the three cultures with initial phosphate concentrations of 400, 200, and 100mg/l, respectively. It can be inferred from the previous data (**Fig. 4.22**) that, the lower the RQ value, the higher the alginate production rate of cells.

It should be stressed that the phosphate limited phase differed in the three runs. Generally, after the exhaustion of phosphate in the culture medium the RQ decreased. It either remained relatively stable (at lower initial phosphate concentration of 100mg/l) or became unstable and oscillated (at higher initial phosphate concentrations of 200 and 400mg/l), which may indicate that the bacterium was more sensitive to pO_2 during the phosphate limited phases when the initial phosphate concentration was high (400mg/l), and this sensitivity decreased at lower initial phosphate concentrations (100 and 200mg/l).

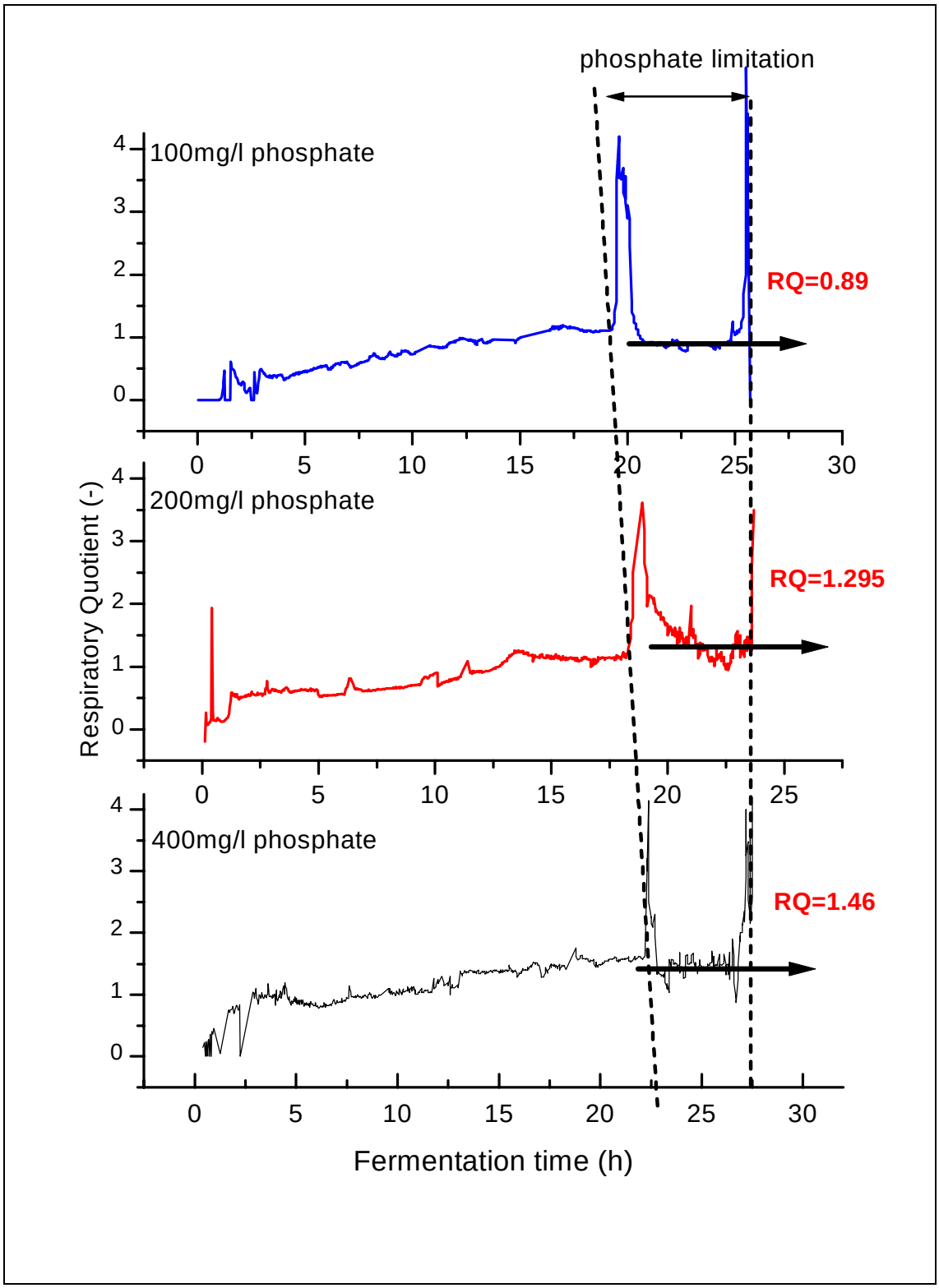


Fig. 4.22 Respiratory quotient as a function of different phosphate levels .

Although culture aeration is generally important for the production of exopolysaccharides of commercial significance, not all microbial cells require maximal aeration for optimal

production. Optimisation of operational variables such as mixing and aeration are particularly important in exopolysaccharide fermentation since high product yield leads to high viscosity of the fermentation liquor, causing a marked decrease in aeration efficiency due to a much lower mass transfer rate between the air bubbles and the broth.

Beside its role as the terminal electron acceptor, O_2 is required in both the synthesis of uronic acid of the biopolymer and indirectly for the reoxidation of the reduced pyridine nucleotides generated. For this reason, RQ is considered to be better than other control variables which are affected by the fermentor geometry (mixing, agitation speed and fermentor hydrodynamics). Thus the aim of the following part was to calculate the theoretical optimum RQ for alginate biosynthesis from sucrose and to confirm this study practically in pO_2 controlled continuous culture.

4.3.2.6. Summary

From the previous results of cultivating *A. vinelandii* at different phosphate concentrations in pO_2 controlled (bioreactor) and uncontrolled batch culture (flasks) it can be concluded that:

- Alginate production by *Azotobacter vinelandii* can not be properly studied in a pO_2 uncontrolled system. This explains the contradictory opinions of different authors in literature in respect to alginate production, because most of their experiments were done in shake flasks or in bioreactor with inaccurate pO_2 control (heterogenous conditions).

- The respiratory quotient RQ may be an additional important parameter for an optimum control of alginate production by this microorganism

4.4 Theoretical consideration:

Optimal RQ for the biosynthesis of alginate and PHB from sucrose

The metabolism of *Azotobacter vinelandii* in relation to alginate and PHB biosynthesis from sucrose was shown in **Fig. 2.4.**

Using NMR studies, Beale and Foster (1996) concluded that hexose in *Azotobacter vinelandii* is entirely catabolized via the Entner-Doudoroff pathway and the triose pools being in equilibrium and reentry into gluconeogenesis prior to alginate synthesis occurs totally from the glyceraldehyde-3-phosphate generated from the Entner-Doudoroff pathway. This pathway obviously utilises a bifurcated route, in which the aldolase reaction produces pyruvate and glyceraldehyde-3-phosphate, the latter can enter gluconeogenesis immediately. So it was reasonable to conclude that only 50% of glucose and 100% of fructose can be directed to alginate formation (Anderson et al. 1987).

Under conditions of high oxygen concentration the tricarboxylic acid cycle (TCA) which consumes the intermediates of glucose and fructose by respiratory pathways is highly active in this strain. The channelling of large amounts of carbon source into the TCA cycle under oxygen stress was proved to be a defence mechanism by which the cell removes extra oxygen to prevent damaging of the nitrogenase system. On the other hand, at very low pO_2 values the carbon source will be mainly channelled into formation of alginate and polyhydroxybutyric acid (PHB).

The main biochemical reactions involved in the metabolism of sucrose and formation of alginate and PHB by *Azotobacter vinelandii* can be expressed as follows:

Catabolism of carbon sources

$$\text{Sucrose} \longrightarrow \text{Glucose} + \text{Fructose} \qquad (4.1)$$

$$\text{Fructose} + 2NAD^+ + 3\ ADP \longrightarrow 2\ \text{Pyruvate} + 3ATP + 2NADH_2 \qquad (4.2)$$

$$\text{Glucose} + 2\ NAD^+ + \ ADP \longrightarrow 2\ \text{Pyruvate} + ATP + 2\ NADH_2 \qquad (4.3)$$

$$\text{Pyruvate} + 4NAD^+ + FAD^+ + GDP \longrightarrow 3\ CO_2 + 4\ NADH_2 + FADH_2 + GTP \qquad (4.4)$$

Alginate biosynthesis

$$\text{Fructose} + \text{ATP} + \text{GTP} + \text{NADP}^{+} \longrightarrow \text{NADPH}_2 + \text{ADP} + \text{GDP-mannuronic acid} \quad (4.5)$$

$$\text{Glucose} + \text{ATP} + 3/2\ \text{NADP}^{+} + ½\ \text{GTP} \longrightarrow 3/2\ \text{NADPH}_2 + \text{ADP} + \text{Pyruvate} + ½\ \text{GDP-mannuronic acid} \quad (4.6)$$

$$\text{GDP-mannuronic acid} \longrightarrow \text{Polymannuronic acid} \quad (4.7)$$

$$\text{Polymannuronic acid} \longrightarrow \text{alginate} \quad (4.8)$$

PHB biosynthesis

$$2\ \text{Pyruvate} + 2\ \text{NAD}^{+} + \text{NADPH}_2 \longrightarrow 2\ \text{NADH}_2 + \text{NADP}^{+} + 2\ \text{CO}_2 + \text{Hydroxybutyryl-coA} \quad (4.9)$$

Electron transport system

$$\text{NADH}_2 + (\text{P/O})\ \text{ADP} + ½\ \text{O}_2 \longrightarrow \text{NAD}^{+} + (\text{P/O})\ \text{ATP} + \text{H}_2\text{O} \quad (4.10)$$

In Equation (4.10) the term $NADH_2$ includes also $FADH_2$ and $NADPH_2$. P/O ratio is the number of ADP molecules phosphorylated per 0.5 mol of O_2 consumed.

RQ is defined as the molar ratio of CPR to OUR which were calculated by UBICON from gas flow rate and exit gas composition.

The respiratory pathways (combination of reactions 4.1-4.4 and 4.10) give a RQ value of 1.0. If the carbon source is surely converted to alginate by combining reactions 4.1, 4.4-4.8 and 4.10 a theoretical RQ value of 0.80 can be calculated. Similarly, for the formation of PHB (by reactions 4.1-4.3, 4.9 and 4.10) a theoretical RQ value of 1.33 resulted. The formation of biomass may slightly affect the RQ value if a release or consumption of reducing equivalents and CO_2 is involved. This was in fact observed in the three fermentation runs with different phosphate concentrations. As the formed biomass increased (with increasing initial phosphate concentrations) the RQ value increased after exhaustion of phosphate from 0.89 (at 100mg PO_4^{3-}) to 1.29 (200mg PO_4^{3-}) and 1.46.(400mg PO_4^{3-}). In addition, the increase in PHB content of the biomass with increasing phosphate concentrations should be considered as another important factor for increasing the RQ value.

It must be pointed out that this theoretical RQ value was also verified from CPR and OUR data obtained by Horan et al. (1983) by *Azotobacter vinelandii* in continuous culture under phosphate limited condition at the same value of dissolved oxygen tension (5%).

4.5. Chemostat culture of *Azotobacter vinelandii* under phosphate limitation at different pO_2 values and different growth rates

A continuous culture was performed using *A. vinelandii* growing in nitrogen free medium under phosphate limited condition. The culture was then aerated with a mixture of nitrogen and air at a total constant flow rate of 1.5 l/min. The agitation speed was set at a constant value (500 rpm). The pO_2 was controlled by a conventional PID controller defined through the UBICON facilities by mixing different ratios of the inlet gasses. The pO_2 was controlled at 1, 2.5, 5 and 10%. The experiment was also performed at different dilution rates of 0.08, 0.15, 0.22, and 0.26 h^{-1} with the help of a calibrated feeding pump to determine the effect of growth rates on alginate production by this bacterium. Steady states were established after 4-5 replacement times when the optical density and the gasses outlet concentrations were stable.

Phosphate limitation was chosen to investigate the relation between RQ and alginate biosynthesis for the following reasons:

- under phosphate limited conditions the specific alginate production rate (q_{alg}) and alginate yield ($Y_{alg/x}$) were high
- lower biomass yield was obtained under this condition and thus minimising the effect of biomass biosynthesis on the RQ obtained.
- lower PHB biosynthesis was always noticed with lower phosphate concentrations.

This was important to correlate the observed RQ value with the alginate biosynthetic process.

4.5.1. Biomass and alginate production as a function of pO_2 in chemostat culture of *Azotobacter vinelandii* under phosphate limitation

The effect of different pO_2 values on biomass generation and alginate production is represented graphically in **Fig. 4.23**. As excepted, both growth and alginate formation were clearly affected by the dissolved oxygen tensions of the culture medium. At the lowest pO_2 value tested (1 %) as well as at the highest value tested (10 %), the biomass as well as alginate formation was minimum as compared to the intermediate values of pO_2 (2.5 - 5% air saturation).

The growth yield ($Y_{X/S}$ on consumed sugar), on the other hand, decreased in a linear manner with the increase in pO_2 from 1 to 10 % air saturation (**Fig. 4.24**). With increasing the dissolved oxygen concentrations in the medium, the sugar was mainly wasted in respiration as will be shown later.

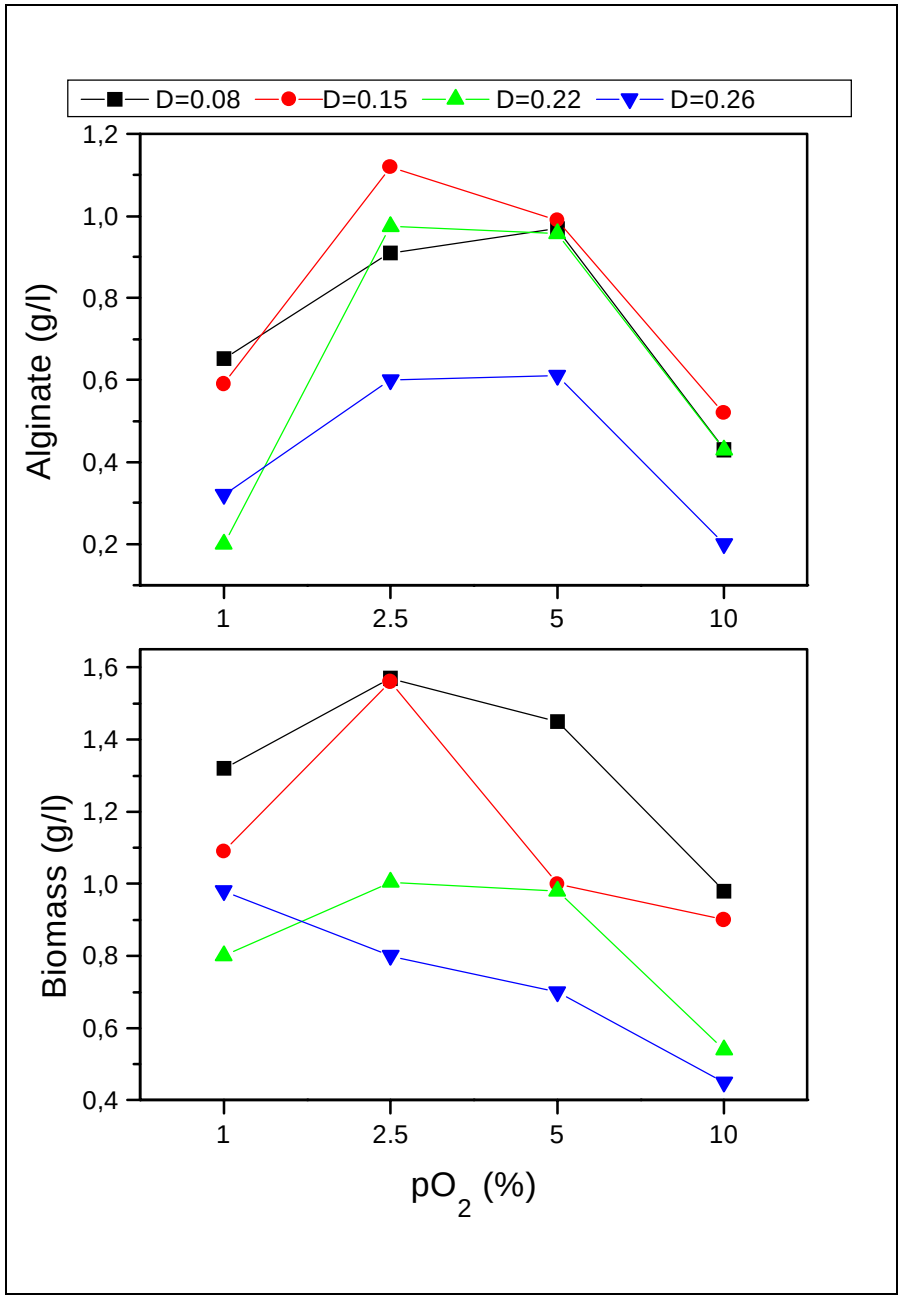


Fig. 4.23 Effect of different dissolved oxygen tensions (1, 2-3, 5 and 10%) on alginate concentration (g/l) and biomass formation (g/l) in phosphate limited chemostat culture of *A. vinelandii* under different dilution rates.

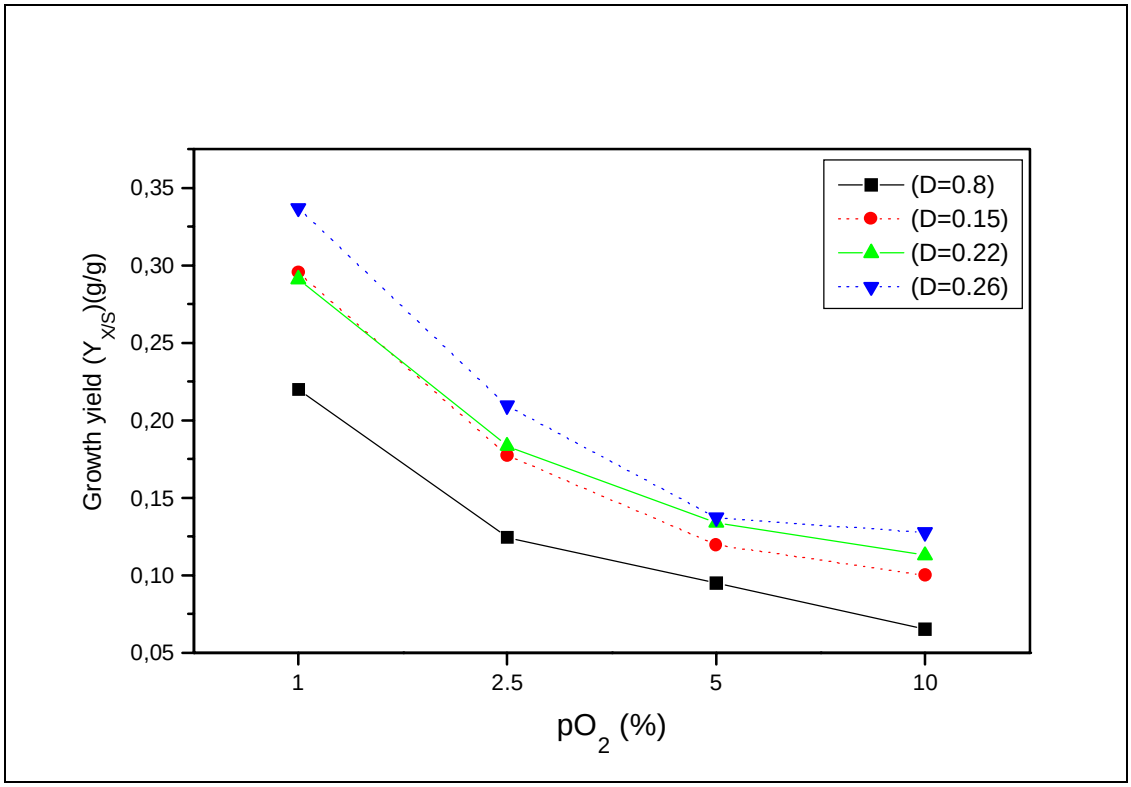


Fig. 4.24 Growth yield ($Y_{X/S}$) as a function of different dissolved oxygen tension and different dilution rates.

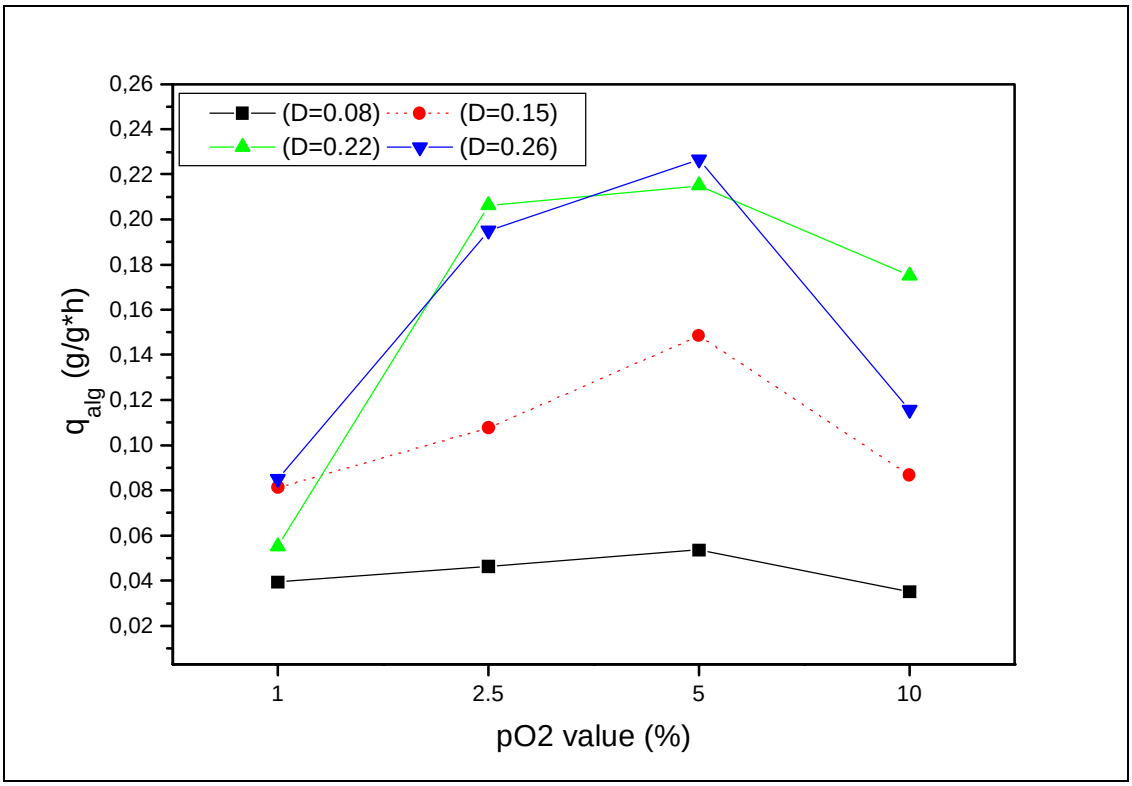


Fig. 4.25 Specific alginate production rate (q_{alg}) as a function of different pO_2 value and different growth rates.

The specific alginate production rate (g/g*h) peaked at the intermediate values of pO_2 (2.5 - 5% air saturation), while at lower and higher pO_2 values it attained lower values (**Fig. 4.25**). It was also obvious that q_{alg} was dependant on the growth rate since it increased sharply with the increase in growth rate till 0.22 (h^{-1}). Parente et al. (1998) reported that the relation between specific alginate production rate and specific growth rate was non linear and dependant on the dissolved oxygen tension. However, this disagreed clearly with the results obtained by Deavin et al. (1977) which showed that $q_{alginate}$ remained relatively constant over a range of growth rates, over a range of respiration rate and with a variety of growth limiting conditions.

4.5.2. Specific substrate uptake rate as a function of pO_2 and dilution rate (extrapolation of m_s) in chemostat culture of *Azotobacter vinelandii*

The results obtained in **Fig. 4.26** strongly suggest that the specific substrate uptake rate (q_s) increased as the pO_2 values increased from 1-10%. This was in fact expected since nitrogenase enzyme is very sensitive to traces of oxygen and in order to keep the amount of intracellular oxygen very low the bacterium wastes the carbon source as CO_2 in respiration and thus protects the nitrogenase system against the toxic effect of oxygen.

Substrates metabolised and not directly leading to biomass or polysaccharide are collectively termed substrate used for maintenance requirement. The substrate used for the maintenance processes (m_s) is derived from plotting q_s against dilution rates and then extrapolating the intersection on the ordinate.

Tab. 4.2 Sugar used for maintenance as a function of different pO_2 levels.

PO_2 %	ms (g/g*h)
1	0,164
2,5	0,345
5	0,376
10	0,826

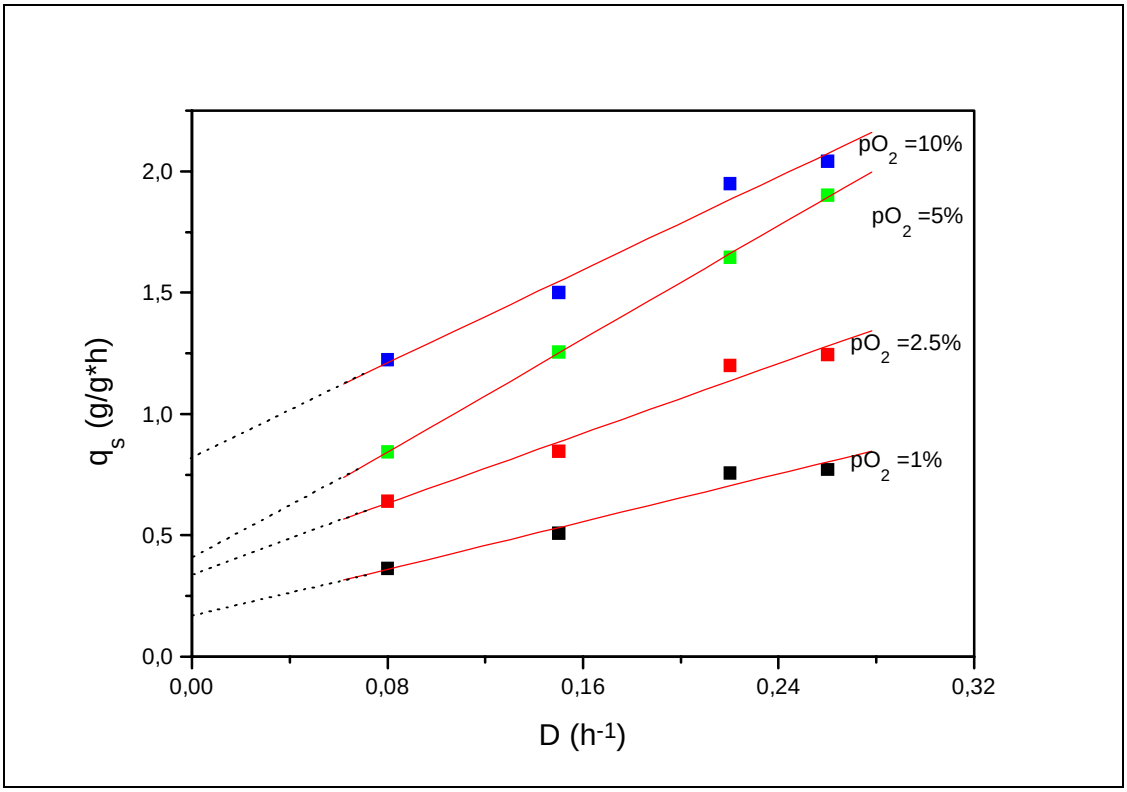


Fig. 4.26 Specific rate of sucrose consumption as a function of dilution rate and the extrapolation of m_s in chemostat cultures of *Azotobacter vinelandii* .

The previous table (**Tab. 4.2**) shows that the sugar consumption for maintenance increased dramatically with increasing the dissolved oxygen concentration of the medium from 1 to 10% air saturation. On the basis of current knowledge it appear that the 'maintenance coefficient' of nitrogen fixing populations of Azotobacter is dramatically different from that of comparable ammonia utilising organisms. Values between 0.5 to 4.7g/g*h were routinely cited in literature with diazotrophically growing cells compared to 0.04g/g*h for ammonia grown cells (Dalton and Postgate, 1969, Kuhla and Oelze, 1988). These very large maintenance coefficents are presumably mainly expressions of 'respiratory protection'.

4.5.3. Respiratory activities:

a) OUR as affected by different pO_2 levels and growth rates in chemostat culture under phosphate limiting condition

The data shown in **Fig. 4.27** demonstrate the evaluation of the effect of pO_2 and growth rates on the specific oxygen uptake rate (mmol/g*h) of nitrogen fixing Azotobacter chemostat culture under phosphate limitation.

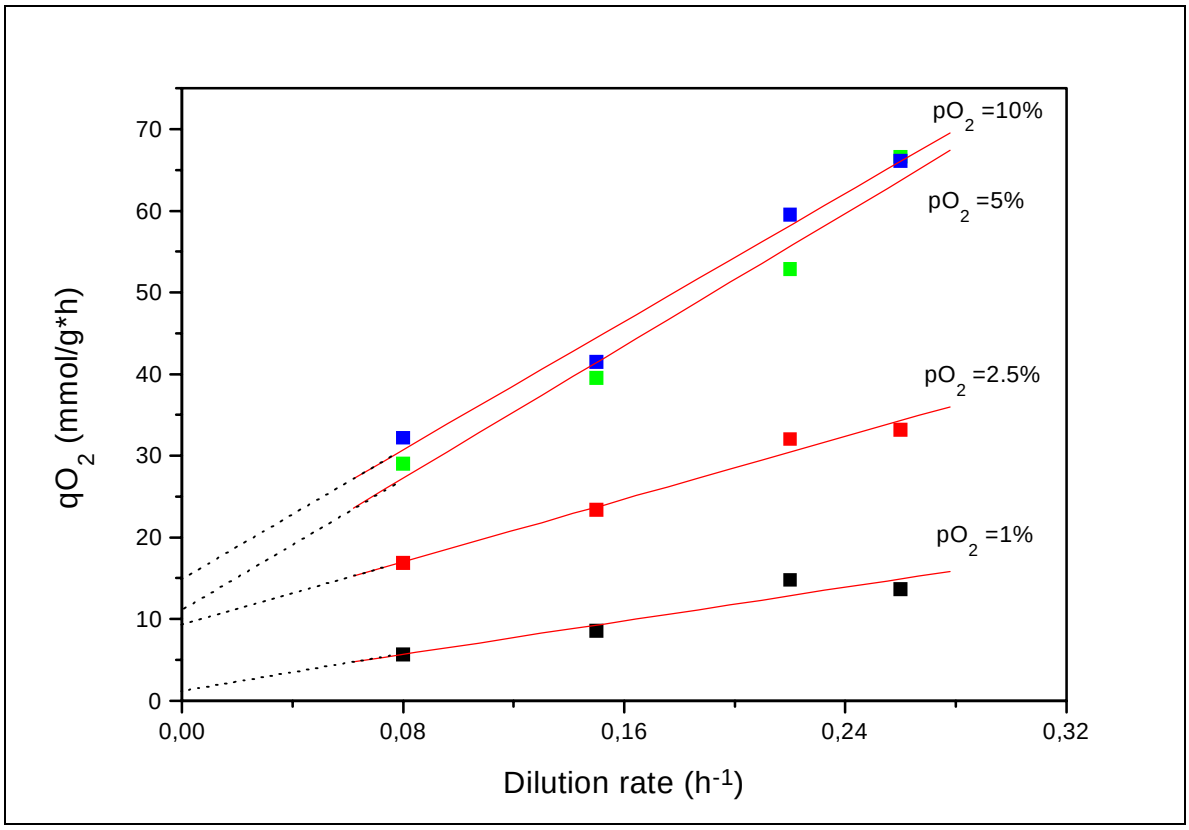


Fig. 4.27 The effect of dilution rates on specific oxygen uptake rate of chemostat culture of *Azotobacter vinelandii*. Extrapolation of m_{O_2}.

From the results represented in **Fig. 4.27** it was found that at a given dissolved oxygen concentration, the respiratory activities increased significantly when D increased. The oxygen consumption rate for maintenance was also calculated by extrapolation. The maintenance oxygen consumption did not increase linearly with increasing dissolved oxygen concentration, but increased sharply till pO_2 of 2.5% (1.6 to 9.3mmol/g*h at pO_2 of 1 and 2.5, respectively) and then slightly increased with the increase of pO_2 till 10% (11 and 15mmol/g*h at pO_2 of 5 and 10%, respectively). Non-linear increase of mO_2 was also reported by the same bacterium under carbon limiting condition (Kuhla and Oelze (1988).

Respiration is an important mechanism in protecting nitrogenase against oxygen damage by lowering the ambient oxygen concentration whenever the capacity for oxygen consumption by the cells exceeds the rate of oxygen input into the culture, and whenever there is enough sugar to be metabolised. It was proposed that this high respiration rate would remove oxygen at the cell surface, and thus creating a compartment of lower oxygen concentration suitable for nitrogenase activities in the interior of the cell.

However, the linear increase in respiration rate in *Azotobacter* does not necessarily mean a linear increase in the energy gained by this organism. Increased respiration in this organism is assumed to be accompanied by uncoupling of electron transport allowing increased oxygen consumption without increasing ATP production (Linkerhäagner and Oelze, 1995). This mechanism in *Azotobacters* protects the cell against high oxygen concentrations.

Surprisingly, the results shown in **Fig 4.27** which are in agreement with the general trend observed by Post et al. (1983) and Boiardi (1994), pronounced that the specific rates of oxygen consumption raised up from 1 to 5% air saturation while remained invariable with increasing the DOT till 10%, results which are not understandable in view of the 'respiratory protection' concept. An unknown function may be then found in addition to respiratory protection. Iwahashi and Someya (1992) postulated that respiratory activity decreases the oxygen concentration, and under decreased oxygen conditions nitrogenase must be protected by energy efficiency to nitrogenase.

b) RQ as affected by different pO_2 levels and growth rates in chemostat culture under phosphate limiting condition

The effect of different pO_2 values on the respiratory quotient profile in chemostat cultures of *Azotobacter vinelandii* under phosphate limitation was evaluated in **Fig. 4.28**.
Independent of the dilution rate, a general trend of RQ was observed against different dissolved oxygen tensions. At pO_2 range of 2-5% of air saturation, the RQ value was very close to the theoretical optimum value (particularly at pO_2 = 5%) as found in batch culture under effective production of alginate. At this narrow range of pO_2 the highest specific alginate production rate was also obtained (**Fig. 4.25**).

The RQ value increased over 1.0 at pO_2 values higher than 5% and lower than 2% of air saturation (except for one steady state at D = 0.26 h^{-1}). The high RQ value at low pO_2 seems to be due to the enhanced formation of PHB and biomass. At pO_2 = 1%, the carbon source converted into biomass and PHB reached 24-37% and 12% of the total carbon consumption respectively, compared to 10.5-15% and no detectable PHB formation at pO_2 = 5% (**Fig. 4.29**). On the other hand, the increase of RQ value at high pO_2 may be attributed to an increased involvement of the TCA cycle (52-60% of the carbon source is converted to CO_2 at pO_2 value of 1% compared to 77-95% at pO_2 of 10%, respectively).

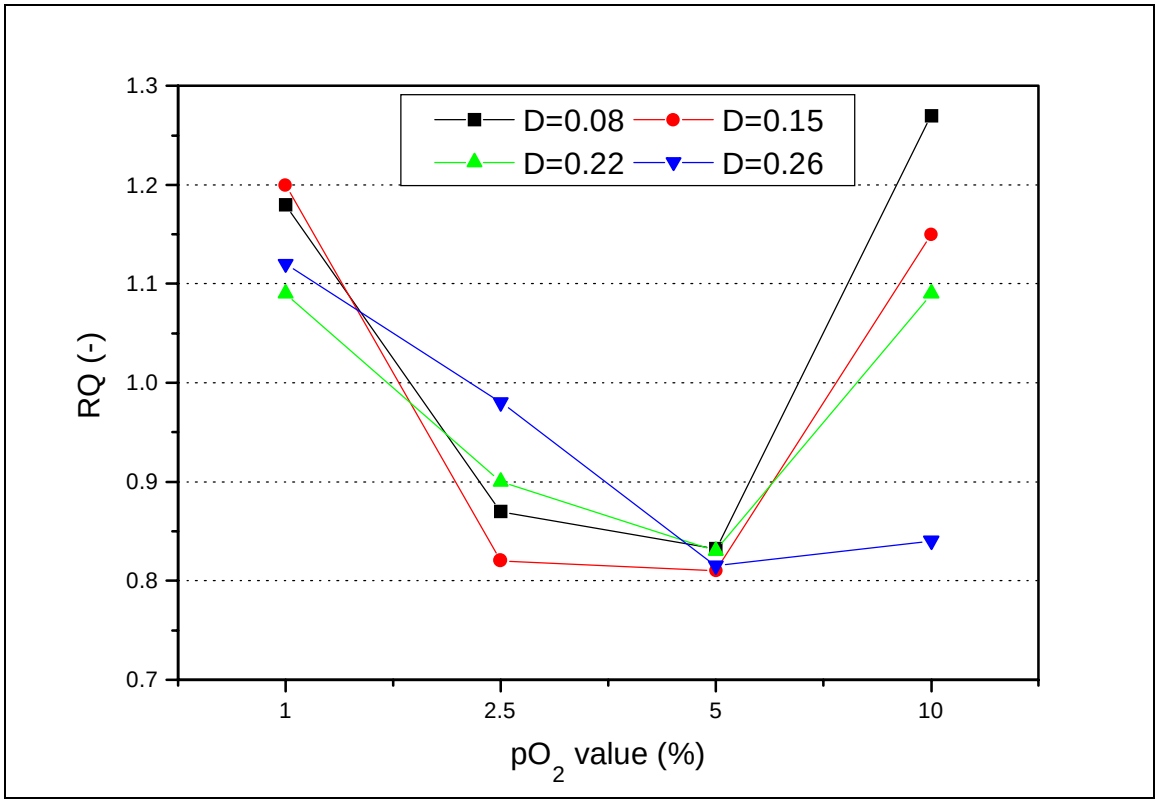


Fig. 4.28 The online measured RQ as a function of different pO_2 values and different growth rates in continuous culture of *Azotobacter vinelandii* under phosphate limited conditions.

Since NADPH derived from biosynthesis and respiration is the primary reductant for the nitrogen fixation in *A. vinelandii* (Sadoff, 1975), an increase in respiration rate as a result of increased dissolved oxygen tension (**Fig. 4.27**) is thus responsible to produce the needed NADPH with the subsequent evolution of CO_2 which results in an increase in the overall RQ values. As pointed out previously (section 4.4), both the formation of PHB and the involvement of TCA cycle can elevate the RQ value in contrast to the situation of alginate formation.

This relatively narrow optimal range of RQ (about 0.8-0.9) may nevertheless represent a challenge from the perspective of process control. Novel control strategies may be still needed for scaling up studies, since this narrow range of RQ comprises a more complicated factor for the success of the process, as in large scale bioreactors due to the inhomogenous oxygen supply, it is difficult to control the pO_2 at very lower values.

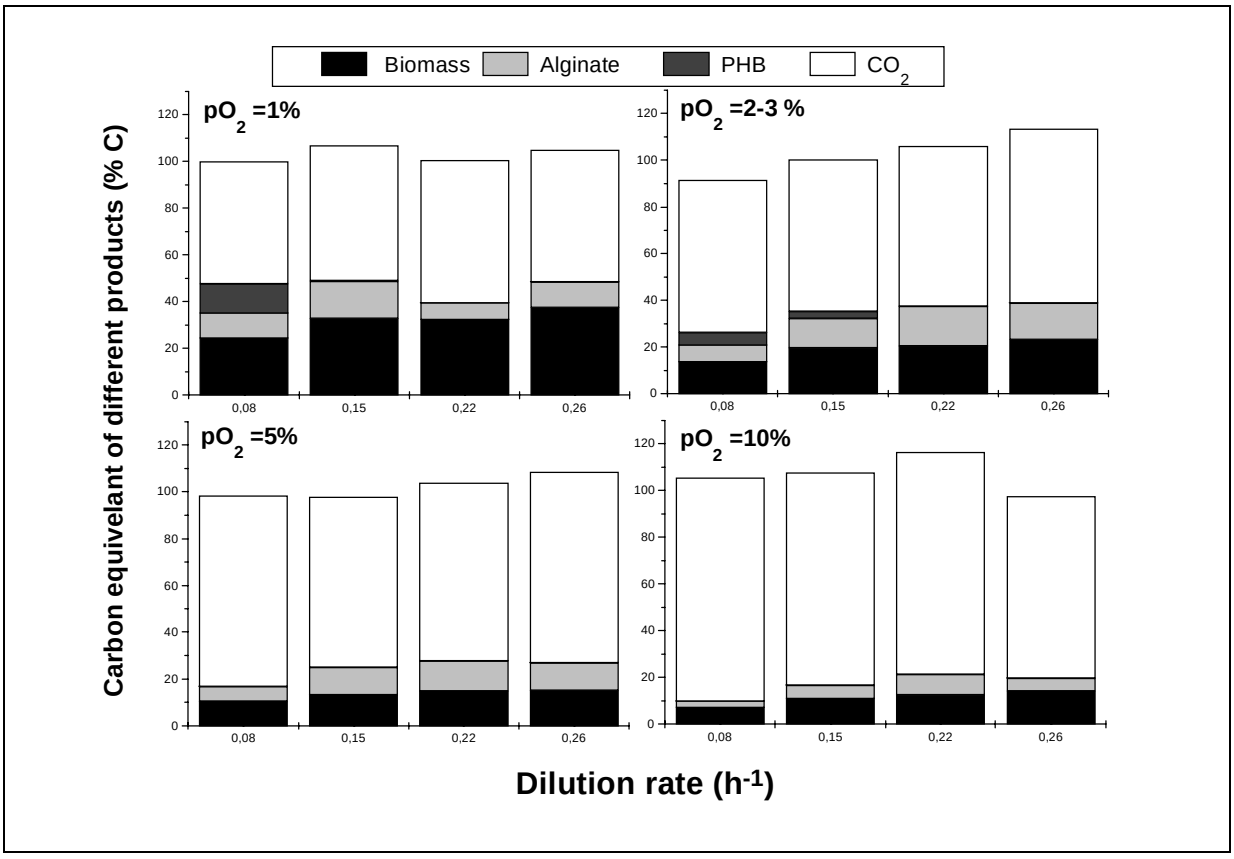
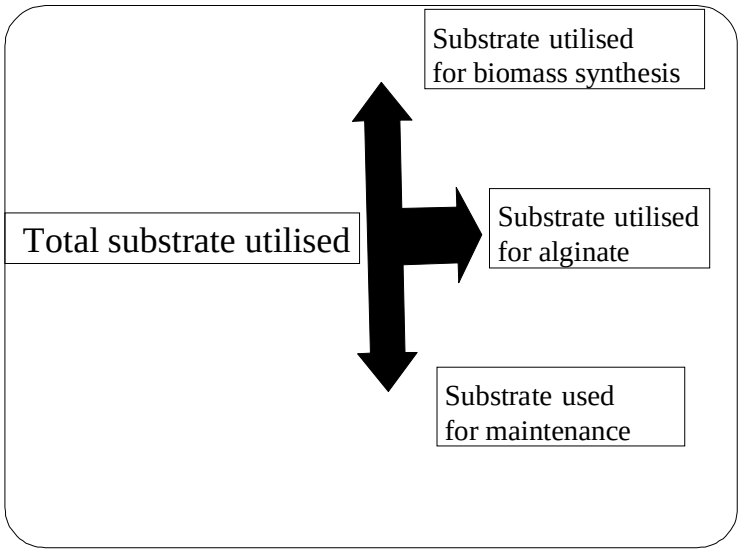


Fig. 4.29 Carbon balance of continuous culture of *Azotobacter vinelandii* as affected by different pO_2 values and different growth rates.

4.5.4. Estimation of assimilated - dissimilated carbon ratio. Calculation of specific substrate uptake rate for maintenance (q_{sm}) at different pO_2 and different growth rates

Sucrose acts as both carbon and energy source for *A. vinelandii*. Estimation of the part of the assimilated carbon source for biosynthesis and that dissimilated to provide energy can be done as follows:



$$\Delta S = \Delta S_{(biomass)} + \Delta S_{(alginate)} + \Delta S_{(m)} \quad (4.11)$$

Assuming that

α is the fraction of carbon in substrate,

β is the fraction of carbon in biomass,

and ϕ is the fraction of carbon in alginate

it follows

$$Y_{(true\ x/s)} = \alpha/\beta = \Delta X/\Delta S_{(biomass)} \quad (4.12)$$

$$Y_{(true\ p/s)} = \alpha/\phi = \Delta P/\Delta S_{(alginate)} \quad (4.13)$$

Where $Y_{(true\ x/s)}$ is the true biomass yield , $Y_{(true\ p/s)}$ is the true alginate yield from sugar.

Rearranging equation 4.12 and 4.13 it follows that :

$$\Delta S_{(biomass)} = \beta/\alpha * \Delta X \quad (4.14)$$

$$\Delta S_{(alginate)} = \phi/\alpha * \Delta P \quad (4.15)$$

Combining equation 4.11, 4.14 and 4.15 and rearranging lead to the following equation:

$$\Delta S_m = \Delta S - \beta/\alpha * \Delta X + \phi/\alpha * \Delta P \quad (4.16)$$

where ΔS_m represents the carbon source used in maintenance or dissimilated carbon source. Similarly $q_{(sm)}$ the specific substrate uptake rate for maintenance (dissimilated carbon) can be calculated as follows:

$$q_{(sm)} = D*S_m/X \quad (4.17)$$

from equation (4.11) and using specific substrate uptake rate.

$$q_{(s)} = q_{(sm)} + q_{(as)} \quad (4.18)$$

where $q_{(as)}$ is specific substrate uptake rate for assimilated carbon (to biomass and to alginate).

Fig. 4.30 shows the ratio of assimilated/dissimilated carbon fluxes as affected by different pO_2 levels and different dilution rates.

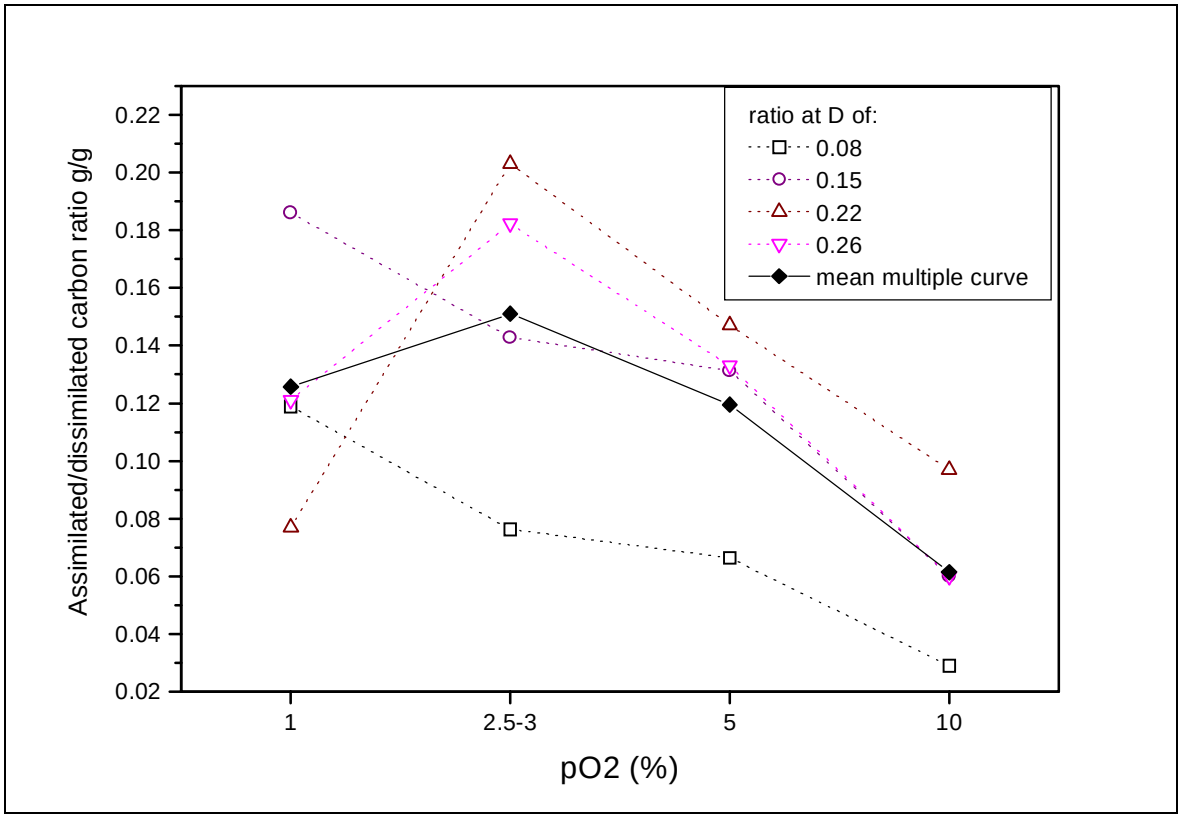


Fig. 4.30 Effect of growth rates and different pO_2 values on assimilated/dissimilated carbon ratio.

The results obtained in **Fig. 4.30** strongly suggest that increasing the pO_2 values to 2.5% resulted in a pronounced increase in the assimilated/dissimilated carbon ratio. However, increasing the pO_2 from 2.5 to 10% air saturation resulted in an increase in the dissimilative pathway through respiration which means an increasing loss of carbon source as CO_2 due to the increase in maintenance requirements for respiratory protection.

4.5.5. The effect of different dissolved oxygen tensions on the molecular weight profile of the produced alginate

Only at the dilution rate of 0.08 h^{-1} the molecular weight of alginate was determined (**Fig. 4.31**). It is clear from the data that an increase in the dissolved oxygen tension resulted in the increase in the relative molecular weight of alginate.

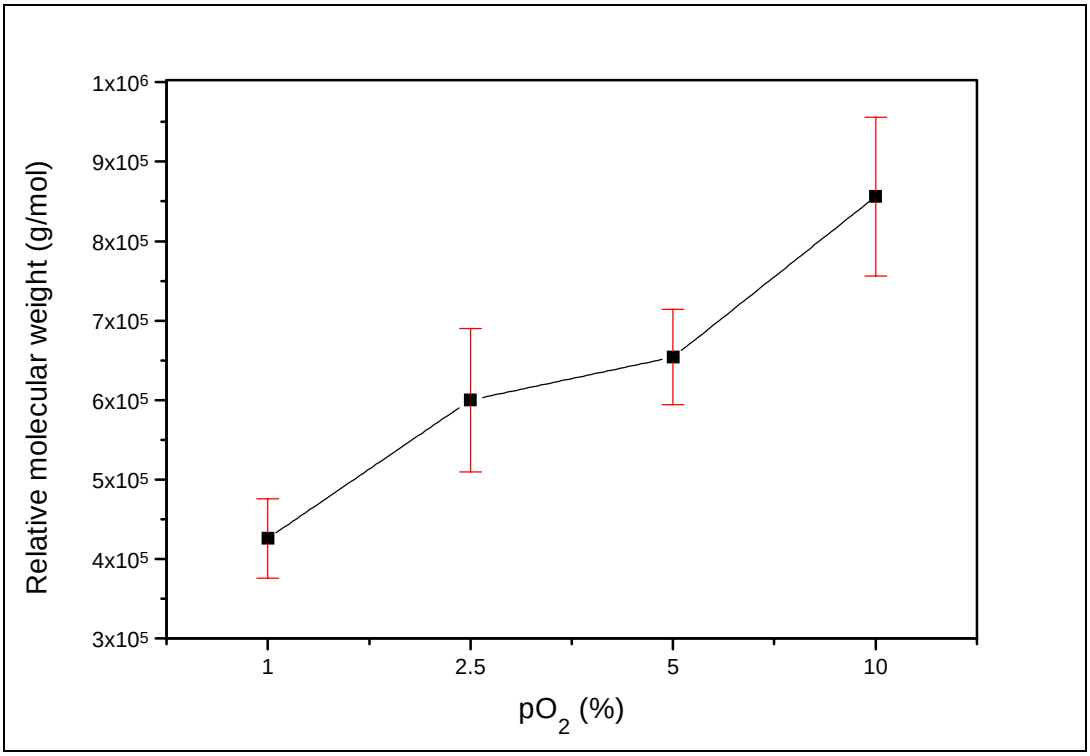


Fig. 4.31 Relative molecular weight of the produced alginate as a function of different dissolved oxygen tensions at constant dilution rate of 0.08 h^{-1}.

These results suggest that the synthesis and/or expression of an alginase could be controlled through the DOT of the culture. The molecular weight of alginate from *Pseudomonas aeruginosa* was also reported to increase with increasing the dissolved oxygen tension of the

culture up to 70% of air saturation (as indicated by the increase in the viscosity of the solution of the isolated polymer) as reported by Leitao and Sa-Correia (1993). It was also concluded by the same author that alginate biosynthesis, especially GDP-mannuronic acid formation, was partially oxygen dependant, and an optimal aeration of 5-10% pO_2 were needed for maximum alginate production while higher aeration (70%) led to a polymer of higher viscosity.

4.5.6. G/M ratio as affected by different pO_2 values

Fig. 4.32 shows the effect of different dissolved oxygen tensions and dilution rates on the guluronic mannuronic acid ratio of the isolated polymer.

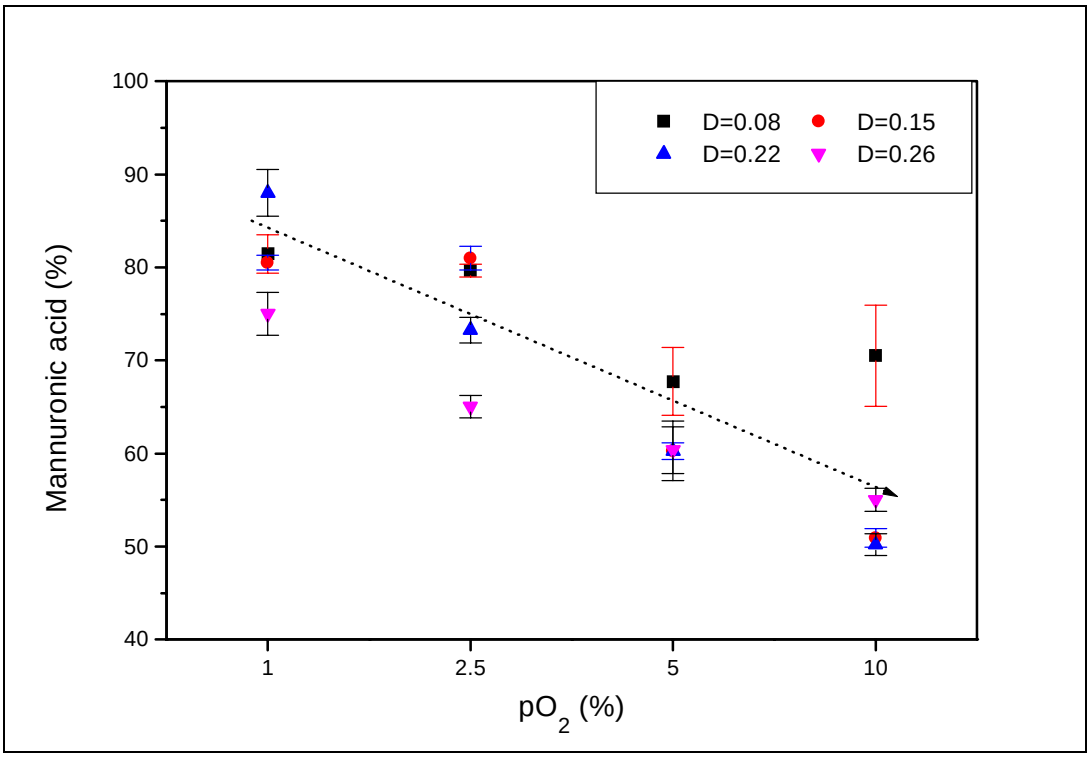


Fig. 4.32 Mannuronic acid content of alginate samples obtained from chemostat culture of *Azotobacter vinelandii* grown at different pO_2 values and different growth rates.

Generally, the guluronic acid residues of alginate samples increased significantly in a linear manner with the increase in the dissolved oxygen tension from 1 to 10%. However, no

marked effect of the different growth rates were observed on the G/M ratio of the alginate samples.

It is worth noting that increasing the molecular weight as well as the guluronic acid content of the alginate samples could be advantageous for the survival of cells in phosphate limiting conditions under oxygen stress. These results can be interpreted as adding further weight to the argument that a harder and more viscous polymer would significantly impede the availability of oxygen, especilaly if the polymer was in the form of a capsule .

4.5.7. Summary

In view of the above facts it can be summarized that a pO_2 range from 2-5% was optimal for alginate production by this strain. Lower or higher values wasted the carbon source either as PHB or in respiration as CO_2. At the optimum range of dissolved oxygen tensions (2-5%), RQ values of 0.8-0.9 were obtained. Those RQ values were close to the theoretical optimal RQ value (0.8) for alginate production calculated for this bacterium using sucrose as carbon source..

The experimental data represented in **Fig. 4.27** strongly suggest an additional yet not described nitrogenase protection system in addition to the two previously reported mechanisms (Section 2.3.4).

Toward the formation of a good quality alginate, in term of molecular weight and a higher guluronic acid residues, it can be concluded that increasing the dissolved oxygen tensions is advantageous for the formation of a high molecular weight and guluronic acid-rich polymer. It is worth noting that the use of RQ as a control parameter as well as the effect of pO_2 on the molecular weight profile of alginate samples were never reported previously on the basis of our recent knowledge.

4.6. Growth with carbon and energy source limitation in a fed batch culture

Fed batch culture refers to the continuous addition of nutrient medium without the withdrawal of any portions of the culture medium resulting in volume variation distinguishing it from the chemostat culture. Since *Azotobacter* is known to have a high respiratory rate, the ability to restrict the substrate feed rate is an advantage when the oxygen transfer rate (K_La) can limit the process rate (Pirt, 1985)

In view of these facts, a fed batch culture was done with *Azotobacter vinelandii* growing diazotrophically to test its growth and alginate production under sucrose (fed substrate) limiting conditions which was then compared with the same sugar quantity in a normal batch culture.

Feeding of sucrose was controlled by its offline measurements during the fermentation run, then the substrate uptake rate (ds/dt) was calculated from linearising the consumed sugar data and a calibrated pump was then used to add the sugar solution with the same rate (**Fig. 4.33**). A limitation of sugar was consequently tested by the further estimation of sugar in the fermentor. The working volume was not dramatically changed during the fermentation run since a very concentrated solution of the fed sugar (500g/l) was used ($\Delta V \approx 0$). In this way, the exponential fed batch became a simple constantly fed batch process with the advantage of less equipment complexity.

Growth of *Azotobacter vinelandii* under sugar limiting conditions increases sharply the oxygen consumption by this strain. **Fig. 4.34** clearly demonstrates the pronounced influence of sucrose limitation on oxygen sensitivity (oxygen uptake rate) and the efficiency of controlling the pO_2 at 5% air saturation by the UBICON system.

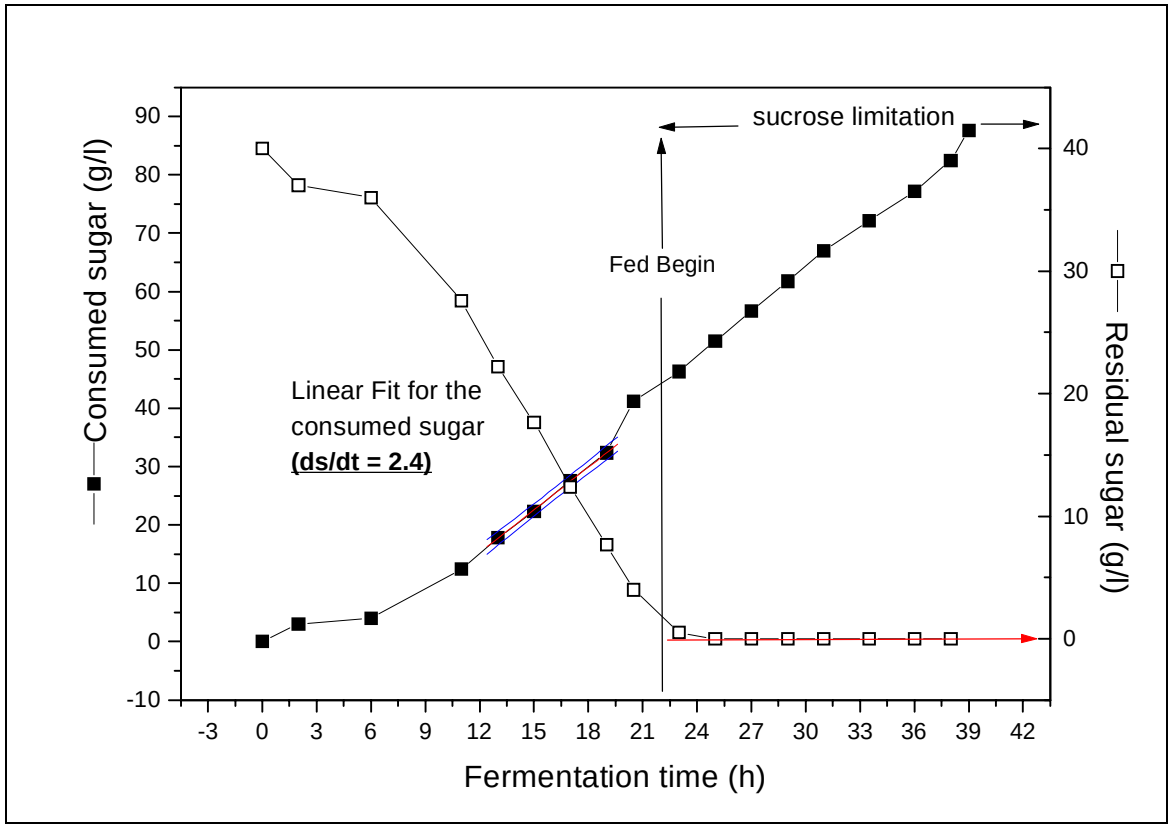


Fig. 4.33 calculation of the substrate uptake rate (Qs) and setting the pump for the desired fed rate.

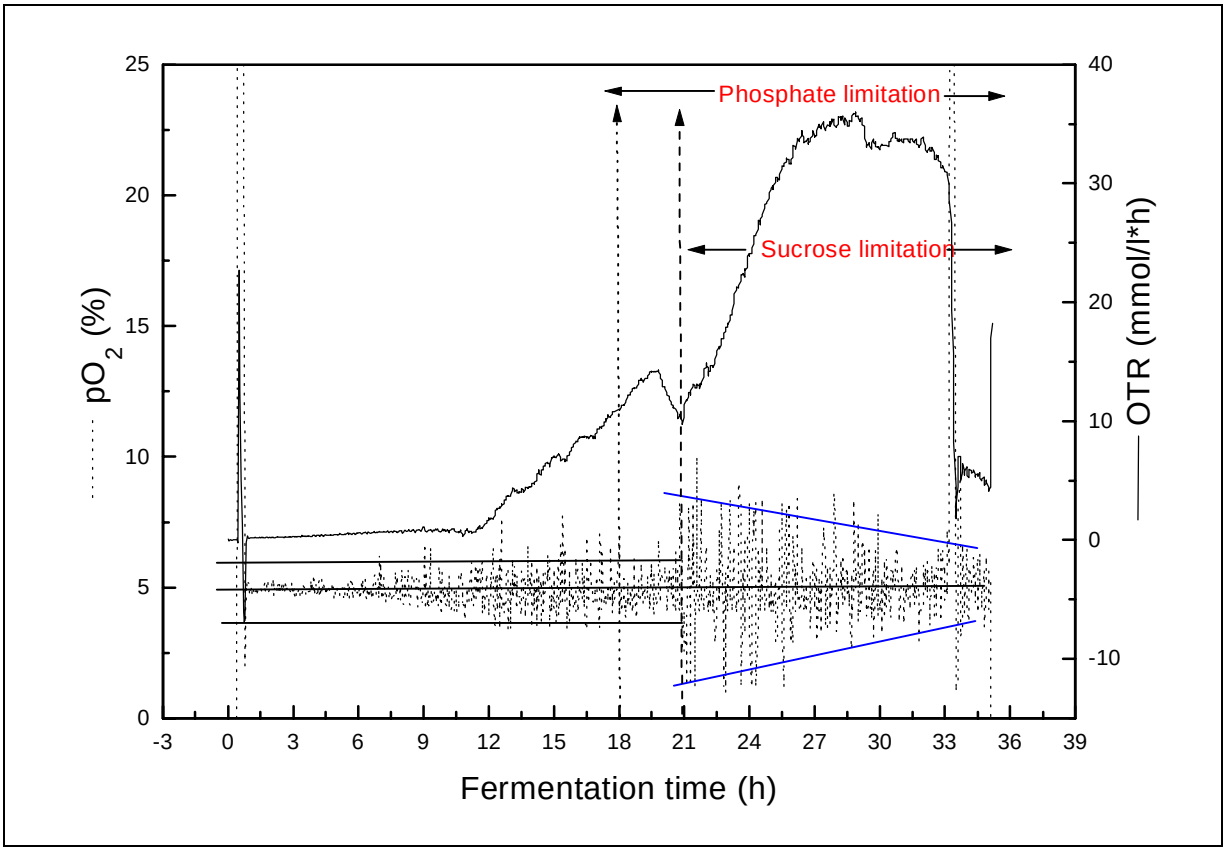


Fig. 4.34 pO_2 control and oxygen transfer rate before and after sucrose feeding.

4.6.1. Biomass and alginate formation

Fed batch mode of growth with sucrose as the fed substrate gave a better biomass concentration of 9.7g/l compared to 8.3g/l with a normal batch process with the same amount of sugar. Surprisingly, sugar limiting conditions gave lower alginate yield, a value of only 3.39g/l alginate was obtained in a fed batch culture compared to 5.8g/l in a normal batch process (**Fig. 4.35**). Alginate yield ($Y_{Alg/X}$), on the other hand recorded its maximum of 0.723 (g/g) after 27h during batch mode compared to 0.384 (g/g) after 39h in fed batch mode. These results strongly support the normal batch process being advantageous in term of alginate quantity.

Based on the fact that alginate is produced even in the presence of sugar limitation, a condition in which the bacterium is expected to make the most efficient use possible of its available carbon and energy substrates to assure its survival, it must than have a survival value.

PHB, on the other hand, was slightly decreased on the onset of sucrose limiting conditions in comparison to that of normal batch which increased in a linear manner with time. However, PHB concentrations in both experiments were low and reached 0.7 and 0.3g/l in batch and fed batch culture, respectively.

4.6.2. Rheological properties as affected by the fermentation mode

In term of polymer quality, however, the polymer viscosity was 2 fold higher than measured in normal batch process. A consistency index of 540mPas with 5.8g/l alginate in a normal batch process was reached compared to 500mPas with 3.4g/l polymer under sucrose limited conditions. For both cultivation systems, n (the flow behaviour index) sinked at the end of fermentation to 0.5 which indicates a pseudoelastic behaviour of the culture medium (**Fig. 4.36**).

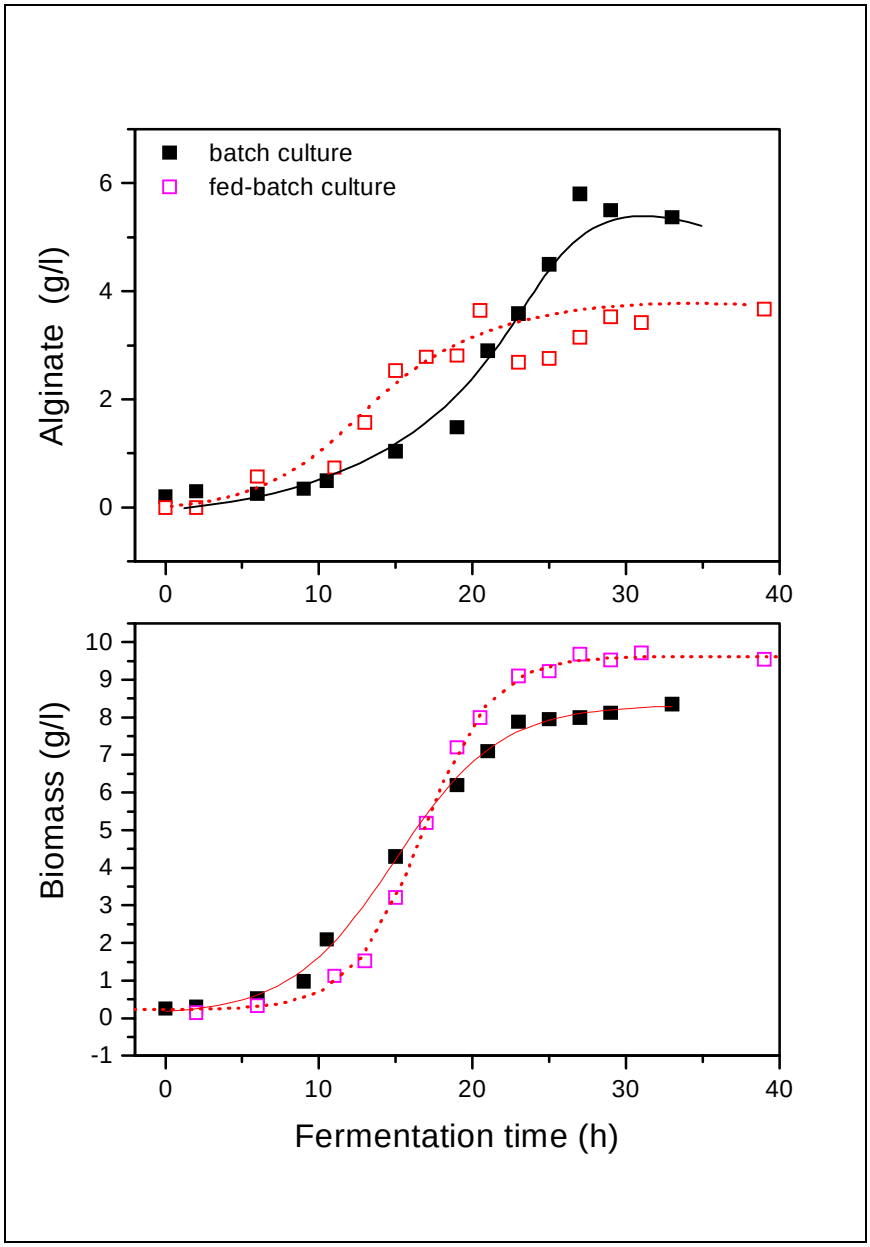


Fig. 4.35 The effect of fermentation mode on biomass and alginate biosynthesis.

For the purpose of precisely comparing the viscosity between the 2 fermentation modes, and to relate the viscosity of the culture to the concentration of the produced alginate a specific consistency index was evaluated as follow:

$$\text{Specific} \quad K = \frac{\text{Consistency} \quad \text{index}}{\text{A lg inate} \quad \text{concentration}} \quad (\text{mPas} / \text{g} / \text{l}) \qquad (4.19)$$

Figure 4.37 shows the specific consistency index as a function of the fermentation mode. It appears that for a fed batch mode the specific viscosity reached a maximum of 160 mPas/g/l

compared to 130 mPas/g/l for batch one, which may indicate that the molecular weight of the polymer produced under sugar limiting condition was higher than that produced during normal batch mode.

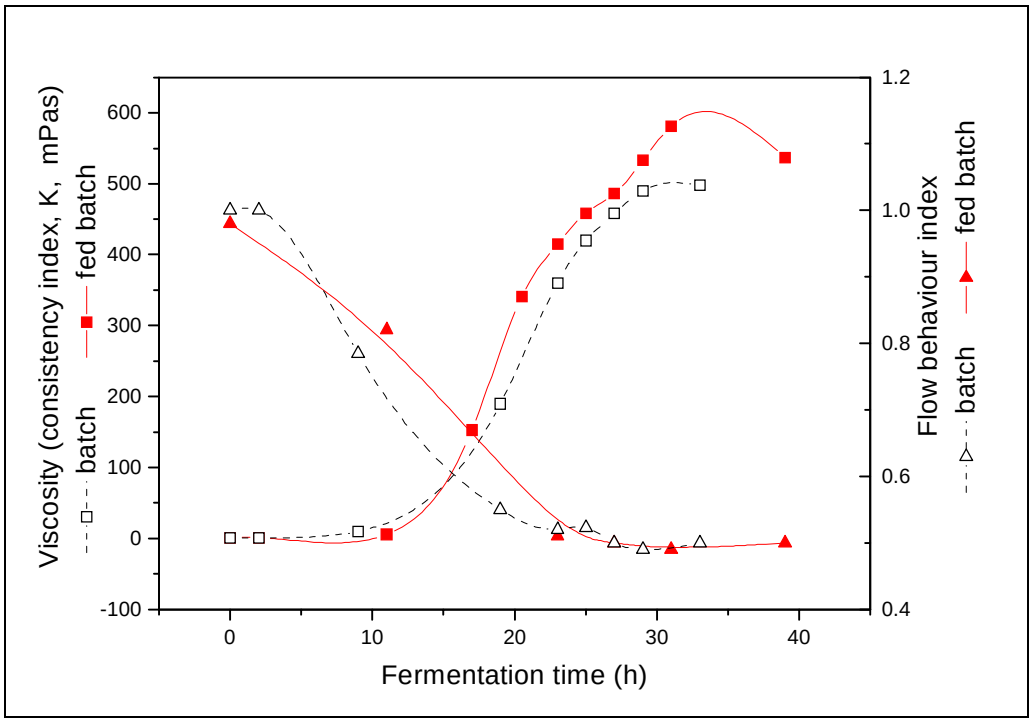


Fig. 4.36 The effect of fermentation mode on the rheological behaviour of the culture medium (Viscosity).

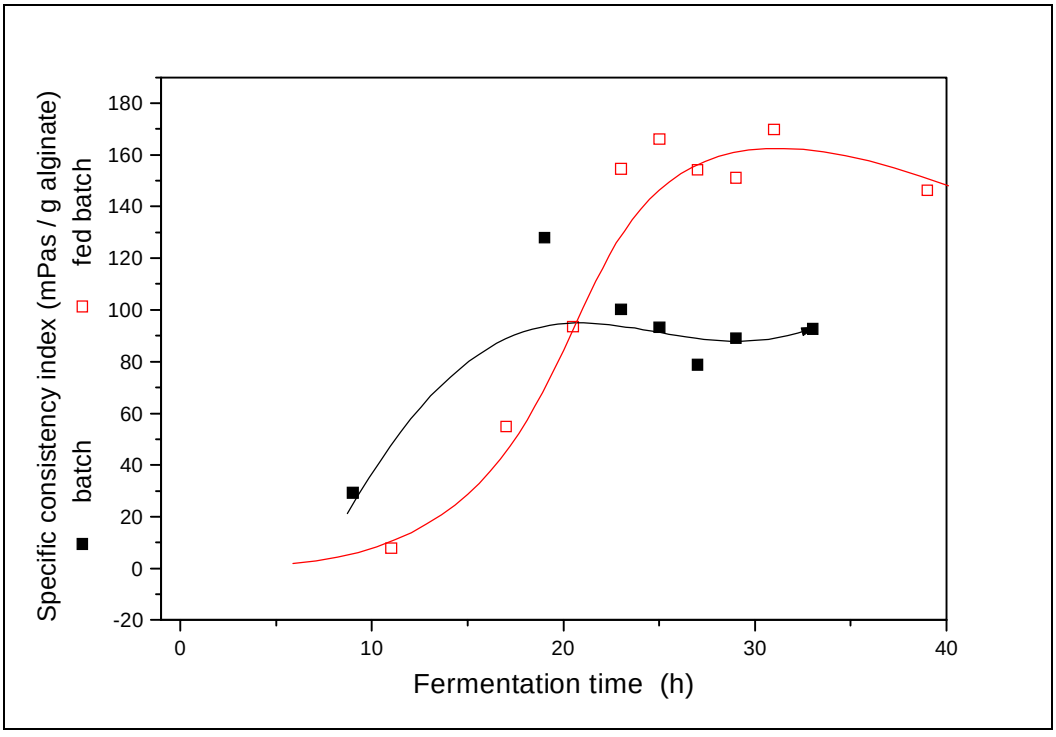


Fig. 4.37 Specific viscosity as a function of fermentation mode.

4.6.2. Quality of the produced alginate as affected by the fermentation mode

In agreement with the viscosity measurements, the molecular weight of the alginate produced under sugar limiting conditions was 2.7 fold higher than that produced under normal batch mode (**Fig. 4.38**). However, in both fermentations the molecular weight profile sinked to a minimum at the end of fermentation (in the decline growth phase) indicating the action of alginate lyase which may be released after cell death.

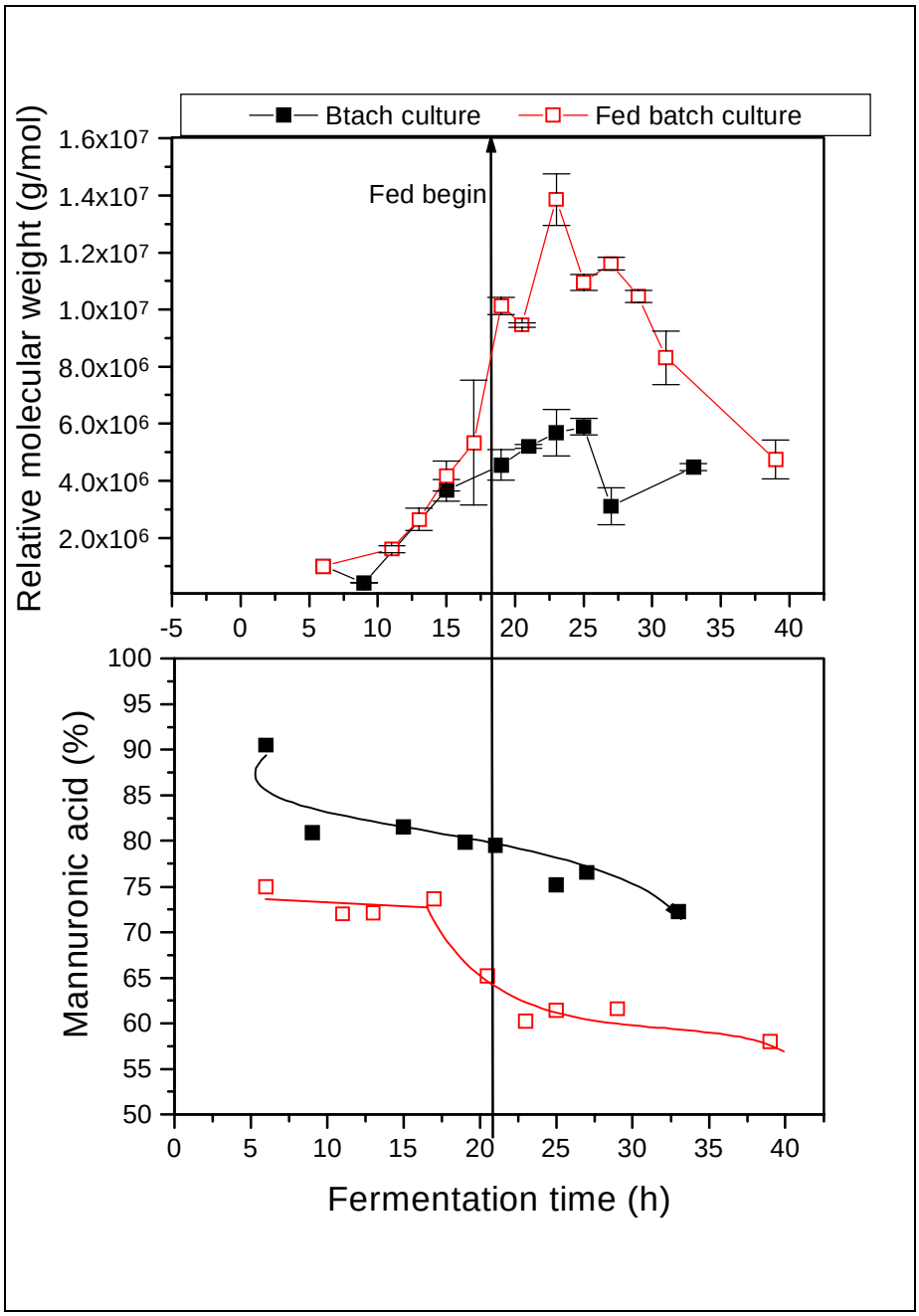


Fig 4.38 Relative molecular weight and mannuronic acid content of alginate as affected by the fermentation mode.

It was also observed that the guluronic acid contents of the produced polymer increased significantly with setting on the fed mode (**Fig. 4.38**). Since gels made from alginate with a high content of guluronic acid have high mechanical strength, low shrinkage, and high stability towards monovalent cations, it appears that a substrate limited growth was favourable for better product quality, in terms of molecular weight and in terms of higher guluronic acid percentage, but not in terms of quantity. However, the higher alginate molecular weight was not accompanied by a harder alginate which disagrees with Gacesa hypothesis (**Gacesa, 1987**) suggesting that the enzymes alginate lyase and epimerase may convert one to another.

4.7. Why does *Azotobacter* biosynthesise alginate?

4.7.1. Theoretical discussion

The biological function of alginate formation in bacteria is not fully understood. Sadoff (1975) has shown that alginate production is important for cyst formation in *A. vinelandii* as a coating protective polysaccharide material (non-capsulate mutants of *A. vinelandii* 12837 were unable to form cysts, Fyfe and Govan, (1983)). This coat protects the cell from desiccation or mechanical stress. Under favourable condition the coat swells and the cyst germinates, divides and regenerates to a vegetative cell. The structure of the microcyst of *A. vinelandii*, however, does not explain the formation of alginate by vegetative cells under conditions not favouring encystment.

A .vinelandii is also a nitrogen fixing organism and since nitrogenase enzyme is inhibited by the presence of oxygen thus one of the priorities of its entire metabolism is to protect the active nitrogenase from being damaged by oxygen. Protection of nitrogenase against oxygen damage has been proposed to occur in this bacterium on the basis of the following two mechanisms: (i) high respiratory activities and thus consumption of the oxygen molecule at the cell's surface and (ii) in case of ineffective respiratory protection, "autoprotection", i.e. the reaction of nitrogenase with oxygen, and reversible inactivation of the enzyme to give a protected yet-inactive state.

Although generally accepted, Post et al (1983) and Boiardi (1994) have questioned the respiratory protection hypothesis. These authors have found that at oxygen concentrations from 30 to 100% air saturation *A. vinelandii* cultures show almost constant respiration rates and negligible decreases in nitrogenase activity, results which are incompatible with the concept of respiratory protection. The contribution of respiratory activity to protect nitrogenase from oxygen damage is still considered a possibility, and it was postulated that energy efficiency was found to be more important as a protective mechanism (Iwahashi and Someya, 1992).

Moreover, respiraory protection for nitrogenase cannot function probably in case of phosphate limitation. And since cells growing diazotrophically in permanent oxygen stress

must have an active nitrogenase system to provide the nitrogen requirements of the cell, the second mechanism is assumed to function only temporally (functions only when other mechanism fails). In view of these facts we strongly suggest that the only way to survive in a phosphate limited culture grown diazotrophically with oxygen stress is to build a viscous slime layer around the cell which may hinder the availability of oxygen.

Adding further weight to this hypothesis are the following observations:

- The morphological studies which were made routinely on culture samples showed the presence of capsules around the cells when grown diazotrophically even in fermentor. The presence of a slime layer or capsule around the cells from agar plates is not surprising since it is a situation of purely diffusional transport of the polymer molecules. However, the slime layer (capsule) did develop even in the presence of high shear rates exerted by the agitation speed in the fermentor (500 rpm).
- The production of alginate takes place mainly in the phosphate limited phase in a pO_2 controlled system (the oxygen stress is always present).
- Based on our results from continuous culture and the results obtained by Post et al. (1983), Boiardi (1994) and in contrast to the 'respiratory protection' hypothesis, the q_{O2} remained constant even when increasing the pO_2 level at higher level (in our study from 5 to 10% air saturation).
- The increase of the polymer viscosity with increasing DOT.
- Production of alginate by A. vinelandii even under sugar limitation, thus increasing the mass transfer resistance of oxygen into the cells.
- Alginate formation is stimulated by the limitation of either iron or molybdenum, which are required for nitrogenase integrity formation (Deavin et al, 1977; Jarman, 1979; Annison and Couperwhite, 1986; Ferrala et al. 1986)
- Growth in the presence of ammonium (the final product of nitrogen fixation) inhibits alginate formation (Brivonese and Sutherland, 1989, and our result).

Based on these observations it is convenient to claim that alginate formation may contribute in combination with the other two mechanisms previously reported in *A. vinelandii* for the nitrogenase protection against excess oxygen (**Fig 4.39**).

However, the protective role of alginate against oxygen does not explain that the optimal pO_2 for alginate production was found to be 2.5-5% and that increasing the pO_2 value decreased the alginate concentration.

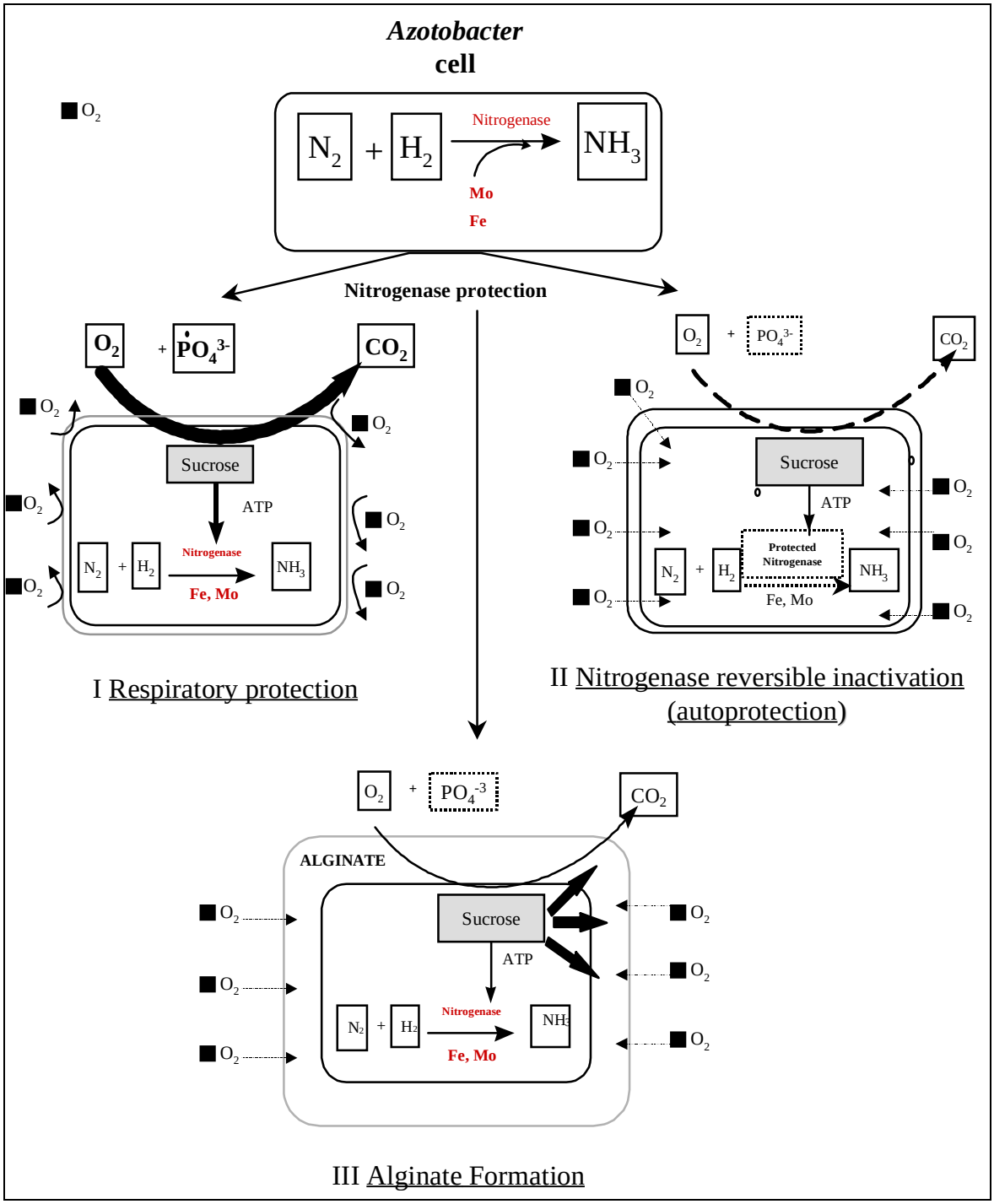


Fig. 4.39 The Nitrogenase protection role of alginate.

The protective role of alginate has been also demonstrated in *pseudomonads*. Selection of growth of alginate producing mutants was observed under the stress of antibiotics, or resistance to virulent phage (Govan et al, 1981)

The following questions should be answered:

- Can diazotrophically growing cells of *A. vinelandii* exist without capsules in pO_2 controlled bioreactor even in the presence of very high shear forces.

- Is varying the thickness of the alginate capsule or varying the compactness of the polysaccharide materials in the capsule or both of them responsible for this protection.

- If this assumption is correct, why the formation of alginate was optimised at intermediate pO_2 values (2-5%) and then decrease afterwards.

For testing the validity of this assumption and to answer the previous questions, the following 2 experiments were performed:

- Capsule formation and its thickness was studied in phosphate limited chemostat culture with different agitation speeds (300-1000rpm).

- Structure of the capsule (compactness of the polysaccharide material around the cell) was studied in phosphate limited chemostat culture with lower and higher pO_2 values (2.5 and 20% air saturation)

4.7.2. Effect of increased turbulence on growth and alginate production in phosphate limited chemostat culture.

The presence or absence of alginate capsule around the cells as a function of different agitation speeds was evaluated in phosphate limited chemostat culture using diazotrophically growing cells. Six steady-states were established between 300 and 1000rpm. In all of the steady states the pO_2 of the culture was kept constant at 5% of air saturation by using UBICON. With the help of a balance connected to a withdrawing peristaltic pump and controlled with UBICON the volume of the culture medium was controlled at 2.5l. The chemostat culture was performed at a dilution rate of 0.15(h^{-1}) with the help of calibrated feeding pump and steady states were established after 4-5 replacement times.

Increased agitation or shaking speed were frequently used by many authors for optimizing the aeration rate or for controlling the dissolved oxygen concentrations for alginate production by *A. vinelandii* (Drozd and Postgate, 1970; Jarman et al. 1978; Jarman, 1979; Annison and Couperwhite, 1984; Brivonese and Sutherland, 1989; Savalgi and Savalgi, 1992; Clementi et al 1995; Pena et al. 1997; Parente et al. 1998). However, no data are available on the independent effect of agitation speed and constant partial pressure of oxygen. Moreover, no systematic study has been reported regarding the influence of increased turbulence on cell morphology and capsule formation in *A. vinelandii* grown diazotrophically under phosphate limited conditions.

4.7.2.1. Biomass and alginate production as a function of agitation speed

Since the intensity of agitation influences the transport of nutrients into cells, increased agitation may increase microbial productivity, due to better mixing and the elimination of the so called ‘dead zone’. This was observed as both alginate and biomass concentrations increased with increasing the agitation speed till 600rpm **(Fig. 4.40)**, but beyond this value both alginate and biomass decreased sharply till 1000rpm. By excess turbulence, it is most likely that the decreased biomass and alginate production may be due to damaging cell membranes and limited mass transfer in localized zones (Toma et al, 1991).

The alginate yield on produced biomass $Y_{alg/X}$ reached also its maximum (0.81 g/g) at the same agitation speed and then dropped till 1000rpm **(Fig. 4.40)**.

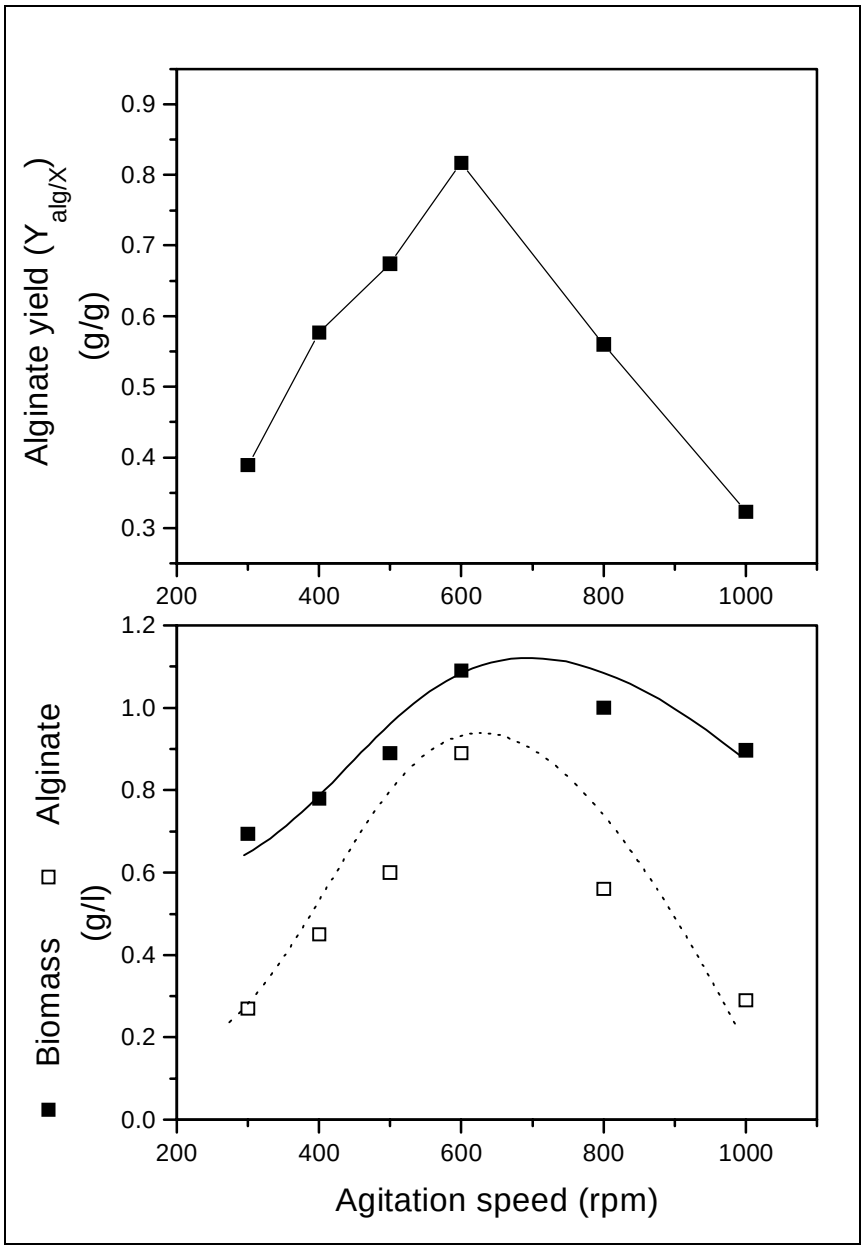


Fig. 4.40 Alginate, biomass concentration and alginate yield as affected by excessive agitation in phosphate limited chemostat culture of *A. vinelandii*.

4.7.2.2. Morphological observations

Surprisingly, even when growing *A. vinelandii* in the excessive agitation speed (1000rpm) capsule formation was observed around the cell which were either circular or ellipsoidal. The resistance of this slime layer to dissolve in the bioreactor medium even in the presence of high shear forces may suggest how important alginate formation is for the survival of this diazotrophically growing bacterium in a phosphate limited and pO_2-controlled microaerophilic culture.

These observation are microscopically presented in **Fig 4.41** where comparative negatively stained preparations of *Azotobacter* cells growing at 600rpm (the optimal agitation speed for both alginate and biomass production) and 1000 rpm (the highest agitation speed used) are viewed.

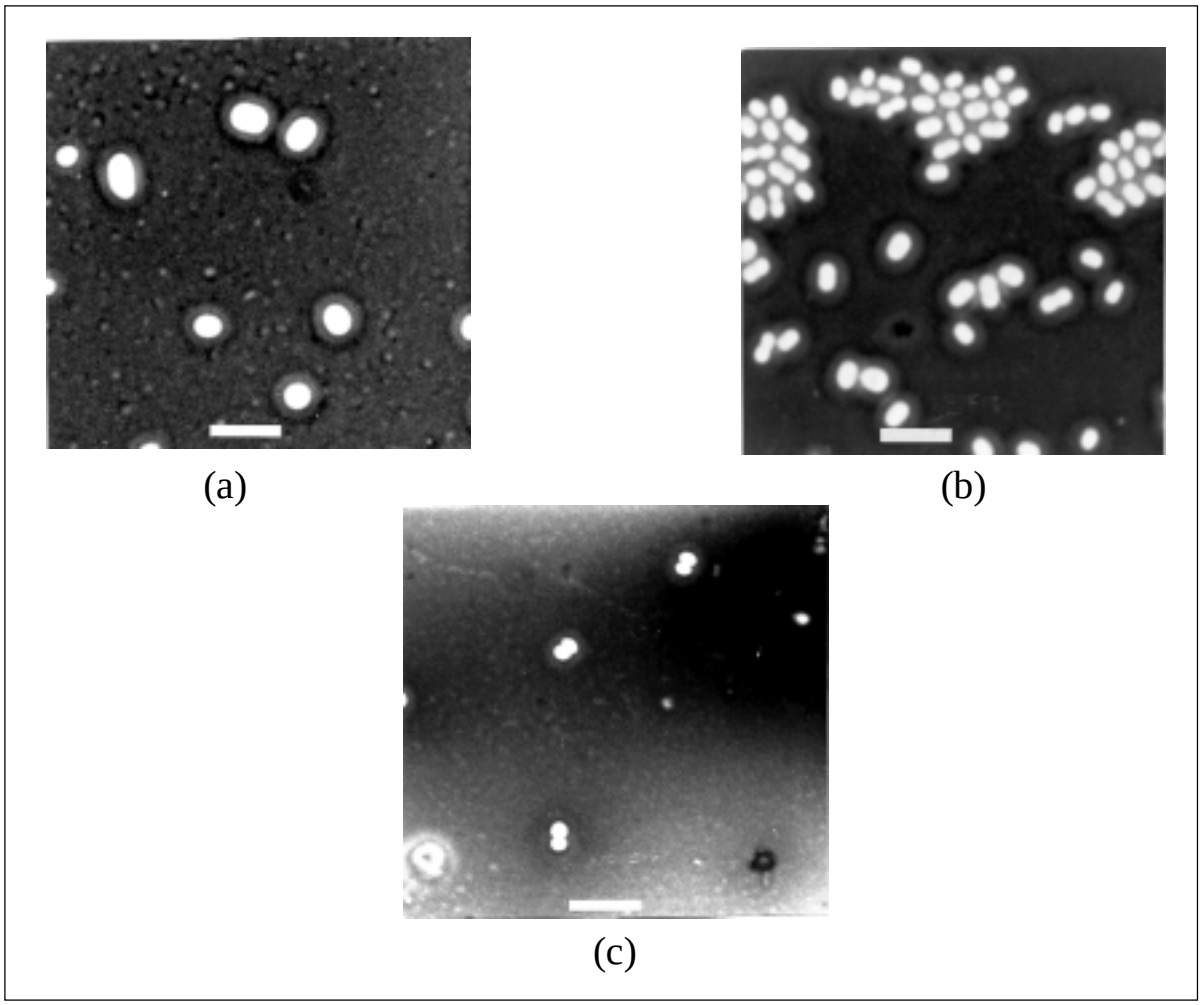


Fig. 4.41 Comparative light microscopic examination of negatively stained *Azotobacter vinelandii* cells growing diazotrophically in pO_2 controlled bioreactor at agitation speeds of (a) 300rpm (the lowest agitation speed applied), (b) 600rpm (the best speed for maximum alginate production) and (c) 1000rpm (the maximum agitation intensity applied). (bar = 0.1mm).

With the increase of shear force, the cell diameter decreased sharply in a linear manner and reached their minimum at 1000rpm of 1.9µm compared to 3.8µm at 300rpm agitation speed (**Fig. 4.42**). Surprisingly, this was not accompanied by a linear decrease in biomass (**Fig. 4.40**) which peaked at 600rpm (1.1g/l). This indicates that the cell numbers increased sharply with increasing the shear stress till 600rpm.

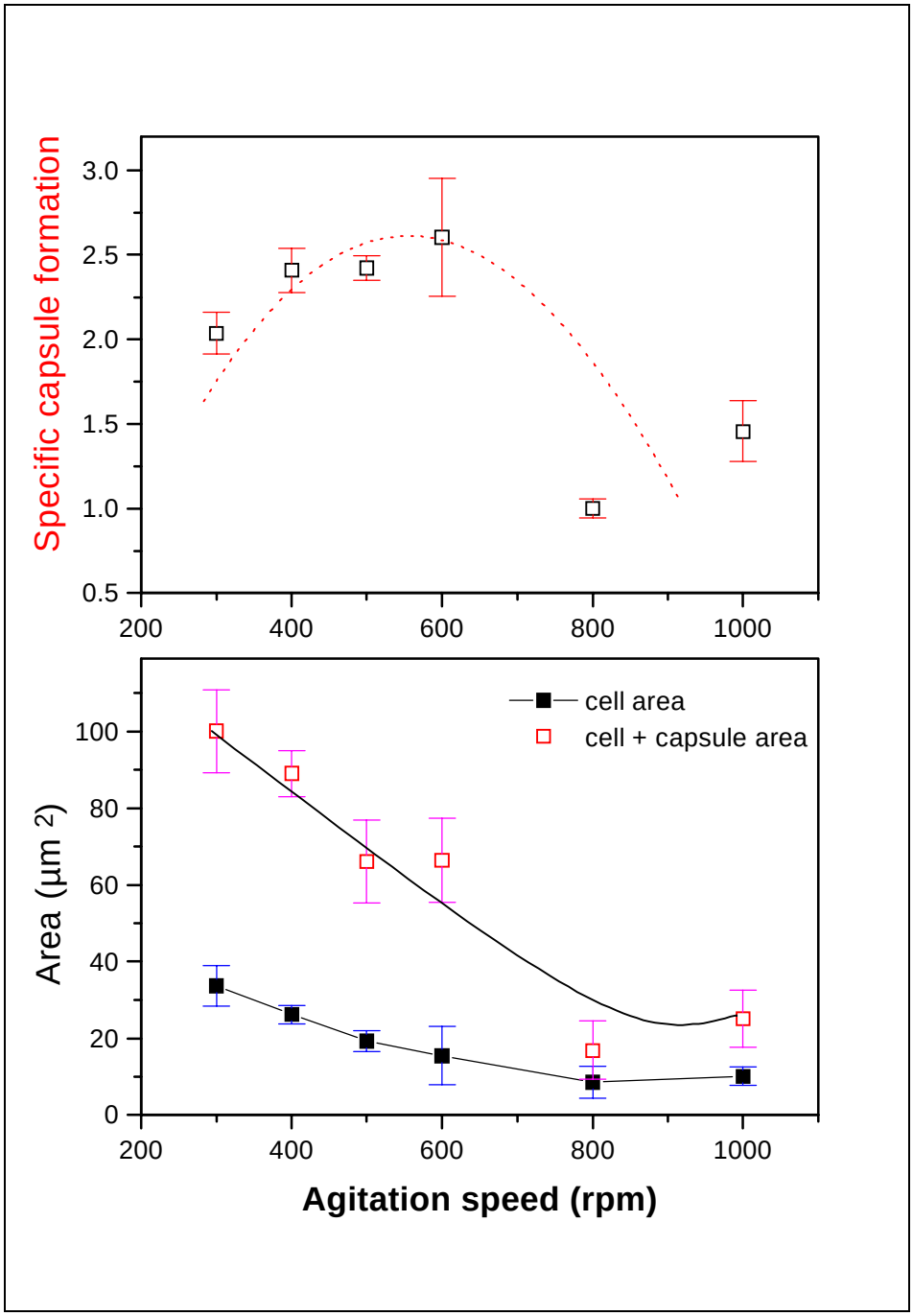


Fig. 4.42 The effect of agitation speed on cell diameter, capsule diameter and specific capsule formation in phosphate limited continuous culture of *Azotobacter vinelandii*.

However, to relate the capsule diameter with that of cell diameter, the specific capsule formation was postulated as follows:

Specific capsule formation = capsule area / cell area (4.20)

[For circular cells the area $= 4 \pi r^2$ where 2r is the radius of the cell and for an ellipsoidal cell the area $= 4 \tau r^*l$ where 2r and 2l are the 2 radiuses of the cell]

It was also noticed that the specific capsule area increased till 600rpm then it decreased again (**Fig. 4.42**). This, however, was in agreement with the alginate yield (g/g) which has its maximum also at 600rpm (**Fig. 4.40**). This indicate that most of the alginate produced under phosphate limiting condition was produced first as capsular polysaccharide and then due to the shear stress some of this capsular polysaccharide was dissolved in the medium and then biosynthesised again so as to limit the mass transfer of oxygen. This maintain the intracellular oxygen concentration at a value low enough for maintaining the nitrogenase activity and high enough for ATP synthesis and respiration.

4.7.2.3. PHB production as a function of agitation intensity

Fig 4.43 shows the effect of the intensity of agitation on the production of PHB by *A. vinelandii* cells.

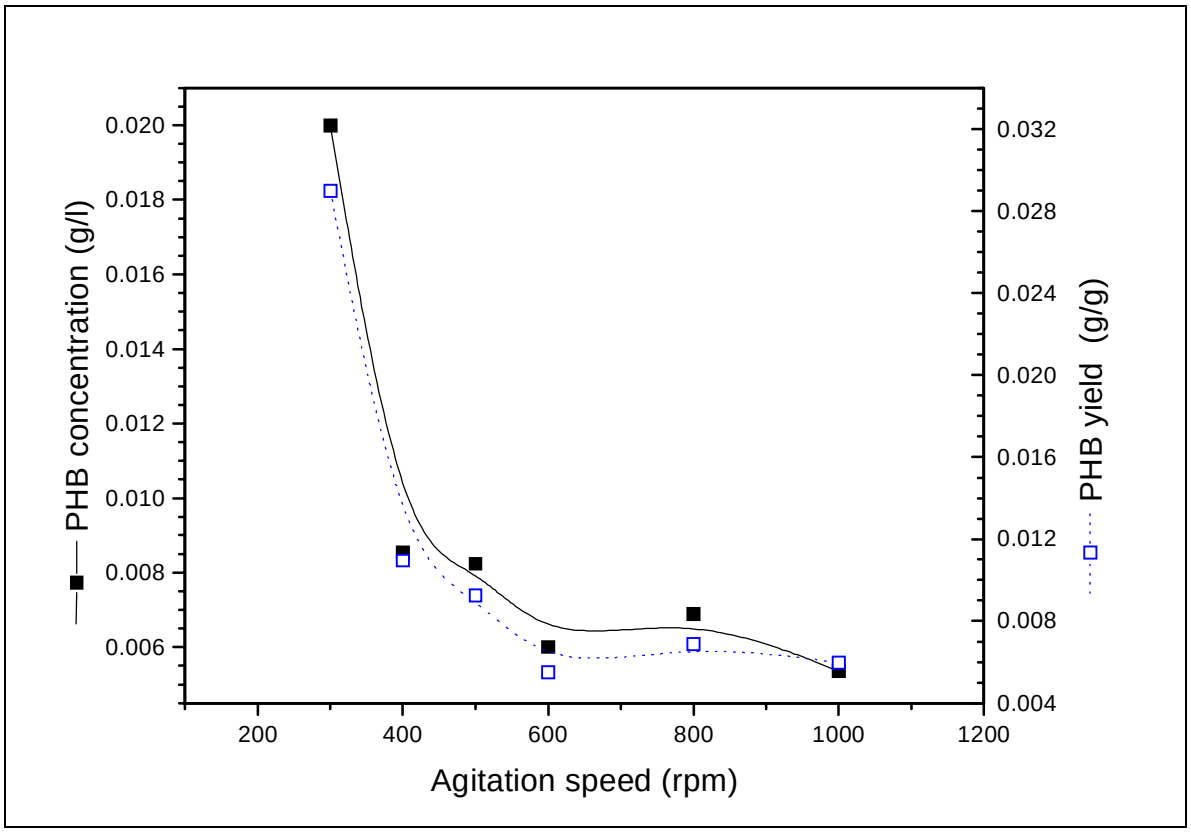


Fig 4.43 The effect of the intensity of agitation on the production of PHB by *A. vinelandii* grown in phosphate limited continuous culture.

The significant linear decrease in the PHB yield with increasing the shear stress may indicate a linear increase in the intracellular oxygen concentration. The relative high concentration of PHB at lower agitation speeds despite the constantly controlled pO_2 values at all of the steady states may indicate that at those agitation speeds stagnant zones with very low mass transfer of O_2 and with very low pO_2 values dominated inside the fermentor. These zones decreases when increasing the agitation speed. This was also confirmed from the alginate yield, which has an optimum agitation speed of 600rpm, at which the optimum effective pO_2 value for alginate production was reached.

4.7.2.4. M/G ratio as affected by agitation intensity

The mannuronic acid content of the alginate polymer isolated at different steady states was found to decrease in amount with increasing agitation intensity (**Tab. 4.3**), in other words, toward the formation of harder capsules resistant to dissolution around the cell.

Tab. 4.3 The mannuronic acid content as a function of different agitation intensity

Agitation intensity (rpm)	Mannuronic acid content (%)
300	80.1
400	83.4
500	78.2
600	72.7
800	60.7
1000	65.9

4.7.2.5. Respiratory activity of diazotrophically growing culture as affected by the agitation intensity

Based on the results represented in **Fig 4.44** the specific oxygen uptake rate (q_{O2}) was shown to increase with increasing the agitation speed. This, however, may reflect the overall effective pO_2 concentration in the bioreactor which is supposed to increase with the agitation speed. The linear decrease of PHB yield ($Y_{PHB/X}$) with agitation speed also supported this

consideration. The RQ profile, on the other hand, decreased with agitation speed, and values around 0.8 (optimal theoretical RQ value for alginate production from sucrose) were obtained at 500 and 600rpm, while at 300rpm and 400rpm higher values were obtained. Higher turbulence (above 600rpm) resulted in a sharp decrease in the q_{CO2} and consequently the RQ values.

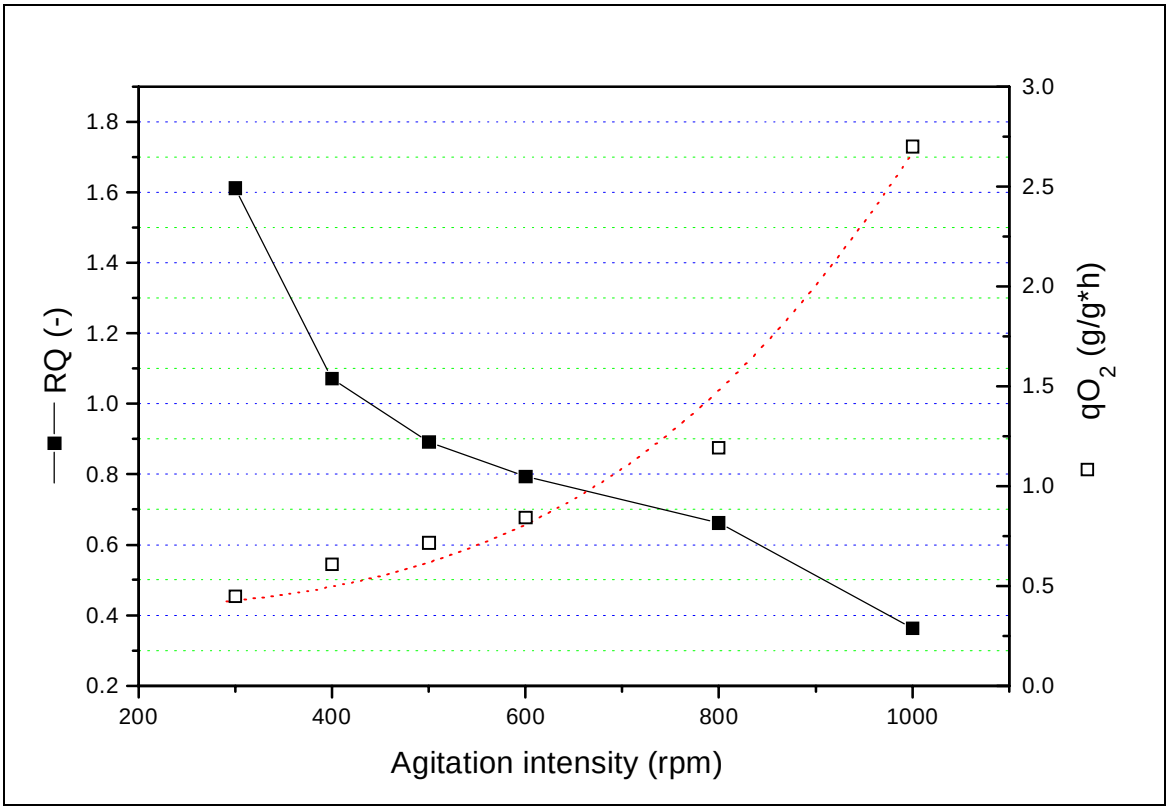


Fig. 4.44 Respiratory quotient and specific oxygen uptake rate as affected by different agitation speed in diazotrophically growing culture of *A. vinelandii*.

4.7.2.6. Summary

It was found that the growth of *A. vienlandii* under diazotrophic conditions was always accompanied by the presence of alginate capsule around the cell.

It is worth noting that on the contrary to xanthan production by *Xanthomonas campestris* and in gellan production by *Sphingomonas paucimobilis*, a non O_2 sensitive strains, samples taken from agar plates did show higher polymer concentrations in the vicinity of the cells (like in case of *Azotobacter vinelandii* colonies in agar plates). However, this layer was easily removed upon shaking and hence no mass transfer resistance in samples taken from the agitated fermentor occurred (Peters et al, 1989; Lobas et al 1992).

Additionally, at higher agitation intensity, the alginate capsular materials were rich in guluronic acid forming harder gel resistant to dissolution. Moreover, this capsule layer was surprisingly not decreased in thickness with the increase in agitation speed which seems not logical.

From the specific oxygen uptake rate data and from the decrease in the intracellular PHB concentration with agitation speed, it can be concluded that the effective overall pO_2 value in the bioreactor increased with increasing shear force, despite of its apparent control by UBICON. However, this was not accompanied with a linear increase in the capsule thickness. Thus no correlation was found between capsule thickness and the extracellular pO_2. And thus the aim of the following experiment was to study, whether the compactness of the alginate capsule differed with differing pO_2 values in a pO_2 controlled chemostat culture with constant stirring speed.

4.7.3. Comparative electron microscopical studies of cells growing diazotrophically at low and very high pO_2 values

This experiment was done to study the effect of different pO_2 values on the compactness of the polysaccharide material around the cell. Two pO_2 values were chosen, the first was 2.5-3% air saturation which proved to be the optimal value for the production of alginate by this strain, and the other was 20% of air saturation.

Diazotrophically growing *A. vinelandii* cells were grown in phosphate limited continuous culture with constant agitation (600rpm) and with varying pO_2 values. The chemostat culture was performed at dilution rate of 0.15(h^{-1}) and steady states were established after 4-5 replacement times.

4.7.3.1. Alginate, biomass and PHB concentration as influenced by pO_2 values

As can be clearly seen from **Tab. 4.4** and in agreement with the previous data (section 4.5), both alginate and biomass concentrations decreased with increasing dissolved oxygen tensions in the bioreactor. The specific alginate production rate (q_{alg}), however, reduced to the half when increasing the pO_2 value to 20% (**Tab. 4.5**). On the other hand, PHB was not detected at a higher pO_2 value while it comprised about 2.1% of the consumed sucrose (based on carbon balance, **Fig. 4.45**).

Tab. 4.4 Phosphate limited chemostat cultivation of *A. vinelandii* at 2.5% and 20% air saturation. Biomass, alginate, PHB formation and consumed sugar.

PO_2	Biomass g/l	Alginate g/l	PHB g/l	Consumed sugar g/l
2.5	0.98	0.715	0.0958	6
20	0.458	0.154	ND	7.25

ND not detected

Tab. 4.5 Phosphate limited chemostat cultivation of *A. vinelandii* at 2.5% and 20% air saturation. Specific production/consumption rate.

pO_2 *(%)*	q_{Alg} g/g*h	q_{PHB} g/g*h	q_s g/g*h
2.5	0.11	0.014	0.93
20	0.05	0	2.37

The specific substrate uptake rate (q_s) increased by a factor of 2.6 with increasing the pO_2 to 20%. On carbon balance basis (**Fig. 4.45**), increasing the pO_2 value from 2.5 to 20% decreases sharply the sugar part entering alginate biosynthesis from 11.7 to 2.08% (5.6 fold), however the cell dry weight was not so drastically influenced by the sudden increase of pO_2 since a decrease of only 2.6 fold was calculated. Till now the decrease in q_{alg} with increasing the dissolved oxygen tensions can not be explained if the protective role of alginate capsule against excess oxygen concentrations is to be assumed.

4.7.3.2. Respiratory activity and RQ

As can be clearly observable in **Tab. 4.6** increasing the pO_2 value from 2.5% to 20% caused a 2.2 and 3.4 fold increase in the specific oxygen uptake rate and carbon dioxide production rate, respectively. On the other hand, an optimal RQ value (0.89) was only observed at the lower pO_2 value (2.5%) which also support the higher specific production rate for alginate (q_{alg}). The same trend was already discussed previously in the present work (Section 4.5.3).

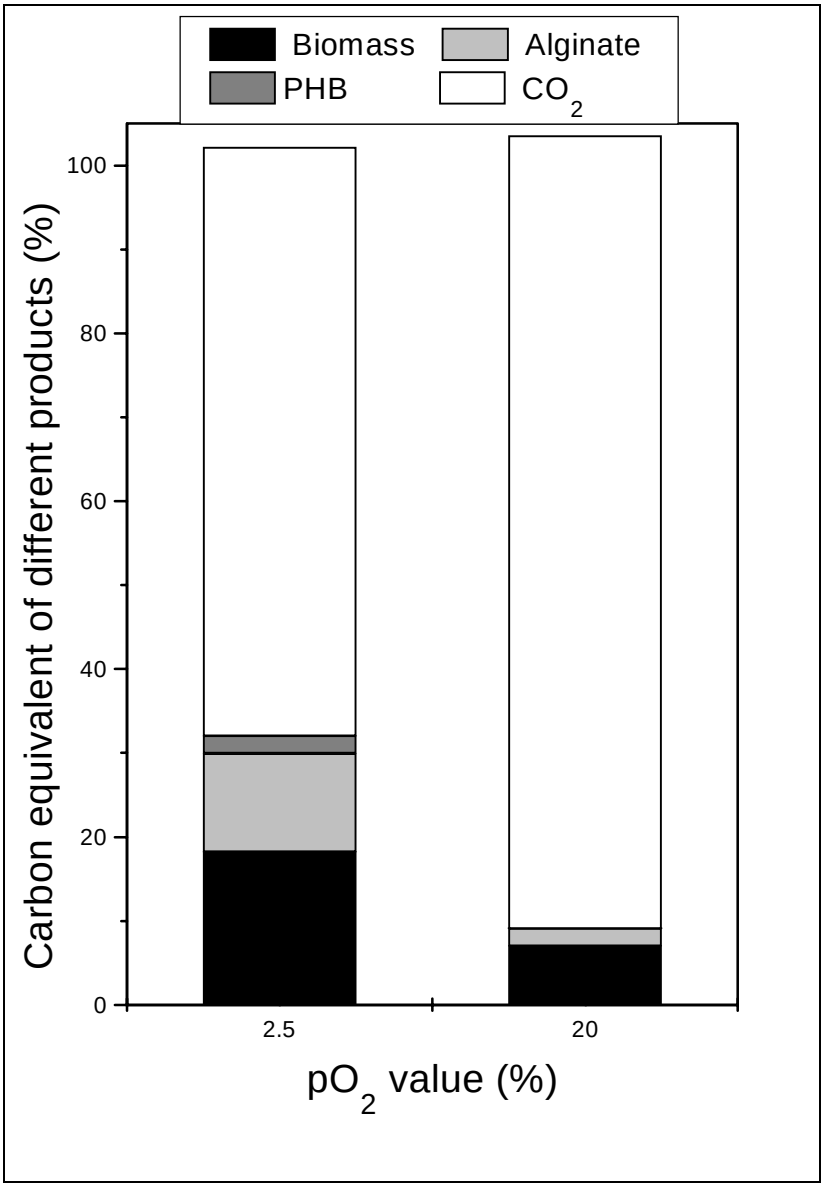


Fig. 4.45 Carbon balance of *A. vinelandii* cultivated in phosphate limited continuous culture at pO_2 of 2.5 and 20%

Tab. 4.6 q_{O2}, q_{CO2} and RQ as affected by 2 different pO_2 values.

pO_2 *(%)*	q_{O2} (mmol/g*h)	q_{CO2}	RQ -
2.5	25.34	22.55	0.89
20	56.15	78.60	1.4

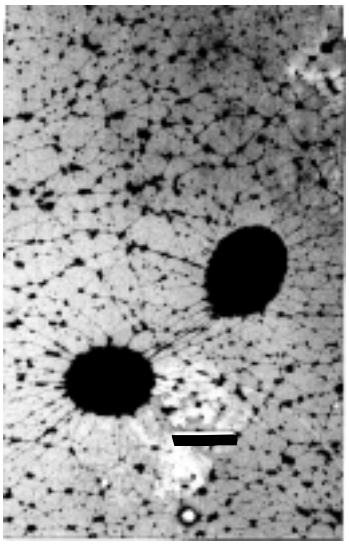
4.7.3.2. Electron microscopy morphological observations

Samples for electron microscopy were taken directly from the fermentor after establishing the steady state. Transmission electron microscopy was used to investigate the alginate concentration gradient around the negatively stained vegetative cell in a surface view as well as in thin section view (see 3.4.12.2)

As is observable in **Fig. 4.46** vegetative cells at both pO_2 values have a net-structured polysaccharide capsule around the cell. However, the structure of polysaccharide in both cases differed forming filamentous structures radiating from the bacterial surface at lower pO_2 value (2.5%) while it formed a compact dense layer of capsular polysaccharide which totally covered the cell membrane and no sign of this filamentous structure was seen.

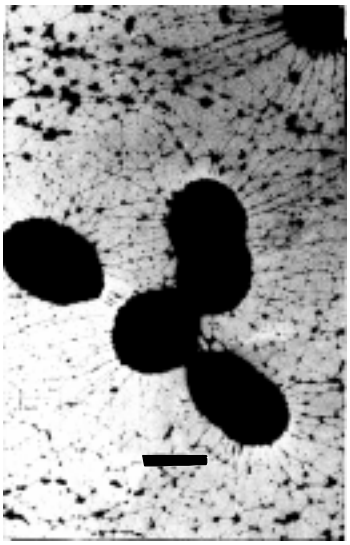
The polysaccharide strands seen radiating from the bacterial surface at lower pO_2 value (2.5%) might indicate the presence of a number of attachments sites. Sutherland (1982) suggested that if those polysaccharide attachment sites are filled, the bacterium produces soluble slime in addition to capsules.

Yet, in view of the experimental data obtained, weight could be added on the hypothesis that the attachment sites are the only exit of the polymeric alginate. At low pO2 levels, the cells is not put under toxic oxygen stress since the nitrogenase protection is assured by the other two mechanisms. In this case alginate strands remain loosely attached allowing the soluble slime layer to be continuously secreted into the cells surrounding medium. On the other hand, elevating the pO_2 value exposes the cell system to the toxic effect of oxygen due to the inefficient nitrogenase protection under phosphate limitation. In this situation the alginate strands exit the cell and remain tightly attached to the cell surface forming the capsule in order to reduce the oxygen mass transfer into the cell. Due to the fact that the attachment sites (alginate exit) are now occupied the overall alginate concentration in the medium decreases since excretion is hindered.

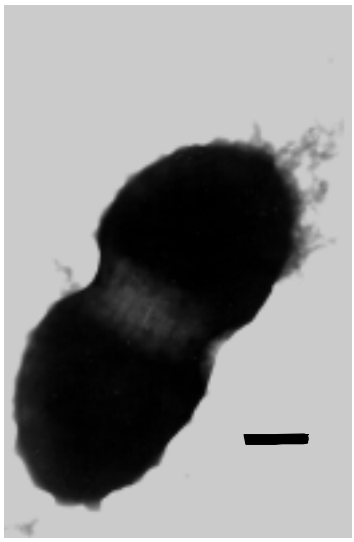


Negatively stained A. vinelandii cells at pO_2 of 2.5% air saturation

(bar=1.7µm)



A Loose net-structured capsule of alginate surround *Azotobacter* cells and filaments of alginate polysaccharides radiates from the cell surfaces



Negatively stained *A. vinelandii* cells at pO_2 of 20% air saturation

(bar=0.6µm)

(bar=1.1µm)

Compact net-structured capsule of alginate surround *Azotobacter* cells and no filaments of alginate polysaccharides are seen

Fig. 4.46 Comparative negatively stained vegetative cells of diazotrophically growing *A. vinelandii* at pO_2 of 2.5% and 20% of air saturation.

It is still not clear, how the alginate exopolysaccharide passed to the outside of the cell and the outer membrane and remains still attached to the bacterial surface to form a capsule. A knob-like element at the termini of many of the polysaccharide filaments was observed from newly synthesized polysaccharide in capsulate E. coli (Sutherland, 1982), but it was not certain whether they played a role in anchoring the polysaccharide to the cell membrane. The

physical relationship of the polymer strands to the cells which produce them has not been studied in the literature.

In addition, the morphological differences in the polysaccharide structure with increasing dissolved oxygen tension are due to the difference in the mannuronic acid to guluronic acid ratio of the alginate around the cell. As obviously observed in **Tab. 4.7** the M/G ratio of the alginate polymer decreased sharply with the increase in pO_2 value of the medium (this was also observed previously, see section 4.5.6). Increasing the guluronic acid residues of the alginate polymer tends to form strong and dense gels with a characteristic interaction between threads of alginate (Egg-box model) while polymers with high mannuronic acid residues form only soft gels with lower affintity for threads binding (Matsumoto et al. 1992). This explains the filamentous and soft structured alginate viewed in case of pO_2 of 2.5% against hard dense gel at 20% air saturation (**Fig. 4.47**).

The regulation of alginate synthesis in *P. aeruginosa* has been intensively studied because of its relevance to respiratory infections in patients with cystic fibrosis (May and Chakrabarty, 1994, Leitao and Sa-Correia, 1997). In contrast, regulation of alginate production in *A. vinelandii* has remained largely unexplored. The biosynthetic gene cluster containing most of the genes coding for the enzymes involved in the synthesis of mannuronic acid, as well as those coding for proteins involved in its polymerization, acetylation, secretion and epimerization are arranged in one operon in *P. aeruginosa*. Whereas in *A. vinelandii algL* (alginate lyase) and *algA* (codes for the bifunctional enzyme phosphomannose isomerase - guanosine diphospho-D-mannose pyrophosphorylase) form part of an operon transcribed independently of the *algD* (codes for GDP- mannose dehydrogenase) promotor.

It was convenient from the previous results to conclude that the dissolved oxygen concentration may regulate some alginate biosynthetic genes. *algL, algA, algC, and algD* which seem either negatively regulated by higher oxygen stress or positively regulated with lower activity of the encoded enzymes and the consequent decrease in the alginate synthesis. Inactivation of alginate enzymes was discussed previously (Leitao and Sa-Correia, 1997).

It is interesting to mention that the mRNA levels of algA, algC and algD genes were found to increase in cells of highly mucoid *P. aeruginosa* grown under increasing dissolved oxygen tension (up to 70%, Bayer et al. 1990; Leitao and Sa-Correia, 1997). Despite of this

upregulation of the alginate genes, however, the activities of the encoded enzymes either maintained or decreased their levels at higher oxygen tensions leading to a slight decrease in alginate synthesis above 10% air saturation.

On the other hand, oxygen-dependant upregulation of *algE1-5* (especially *algE2* which responsible for introducing G blocks) was observed since harder alginate with more guluronic acid residues with the ability to form strong gel was characteristic of higher pO_2 value (**Fig. 4.47, 4.48**).

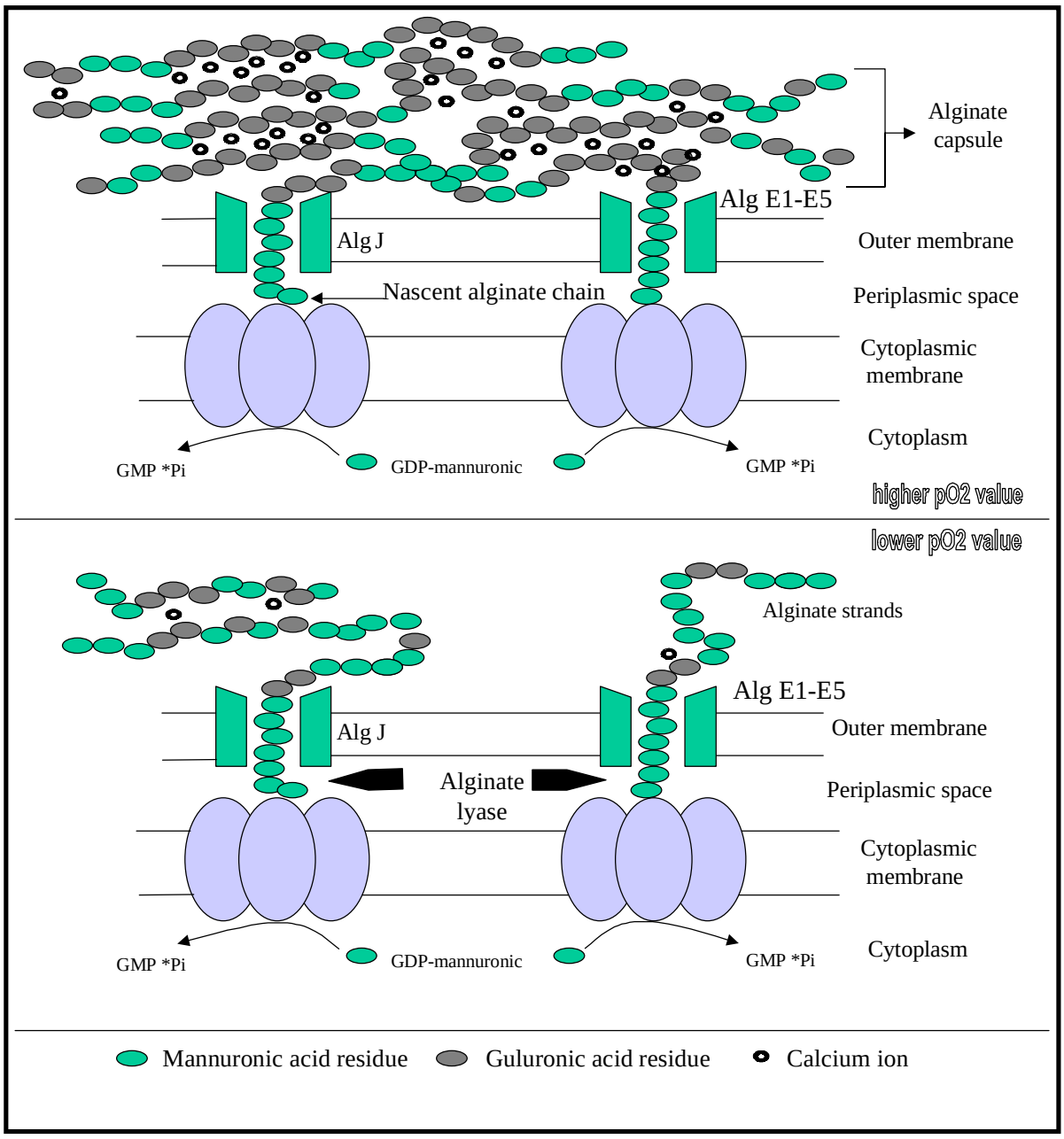


Fig. 4.47 Compact and loose capsule formation in response to high and low dissolved oxygen tension.

Tab. 4.7 the effect of different pO_2 values on the M/G ratio of alginate

pO_2 (%)	Mannuronic acid content (%)
2.5	88.53
20	45.15

Acetylation as well as epimerization provide the cell with a mean to control the compositionand hence physical properties of the produced polymer. However, it is not clear whether *Azotobacter* transacetylase (algI , algF) or its epimerase (algE1-E5) or both of them are responsible for such variation of polymer structure. It is however, important to notice that highly acetylated polymer loose the ability to be epimerised by the epimerases due to steric hinderance.

Fig. 4.48 shows a comparative electron microscopy of thin sections of vegetative cells of *A. vinelandii* grown diazotrophically at different dissolved oxygen concentrations (2.5% and 20%). The significant difference in the compactness of the alginate capsule layer between lower and very high pO_2 value shows how important the physical properties of the alginate capsule (**Fig. 4.47**) are for the mass transfer limitation against oxygen and hence to possibly maintain a stable intracellular oxygen concentration at a lower value suitable for survival.

4.7.3.3. Summary

This experiment was done to test the assumption of the protective role of alginate. In view of this supposition the amount of alginate produced by *A. vinelandii* should increase proportional with the pO2 level. However, the protective role of alginate could be fulfilled either quantitaively by increasing the alginate thickness, i.e. capsule diameter around the cell, or qualitatively by varying the physical properties of the alginate capsule, i.e. the G/M ratio, which results in a more compact polymer layer with a lower O_2 mass transfer efficiency.

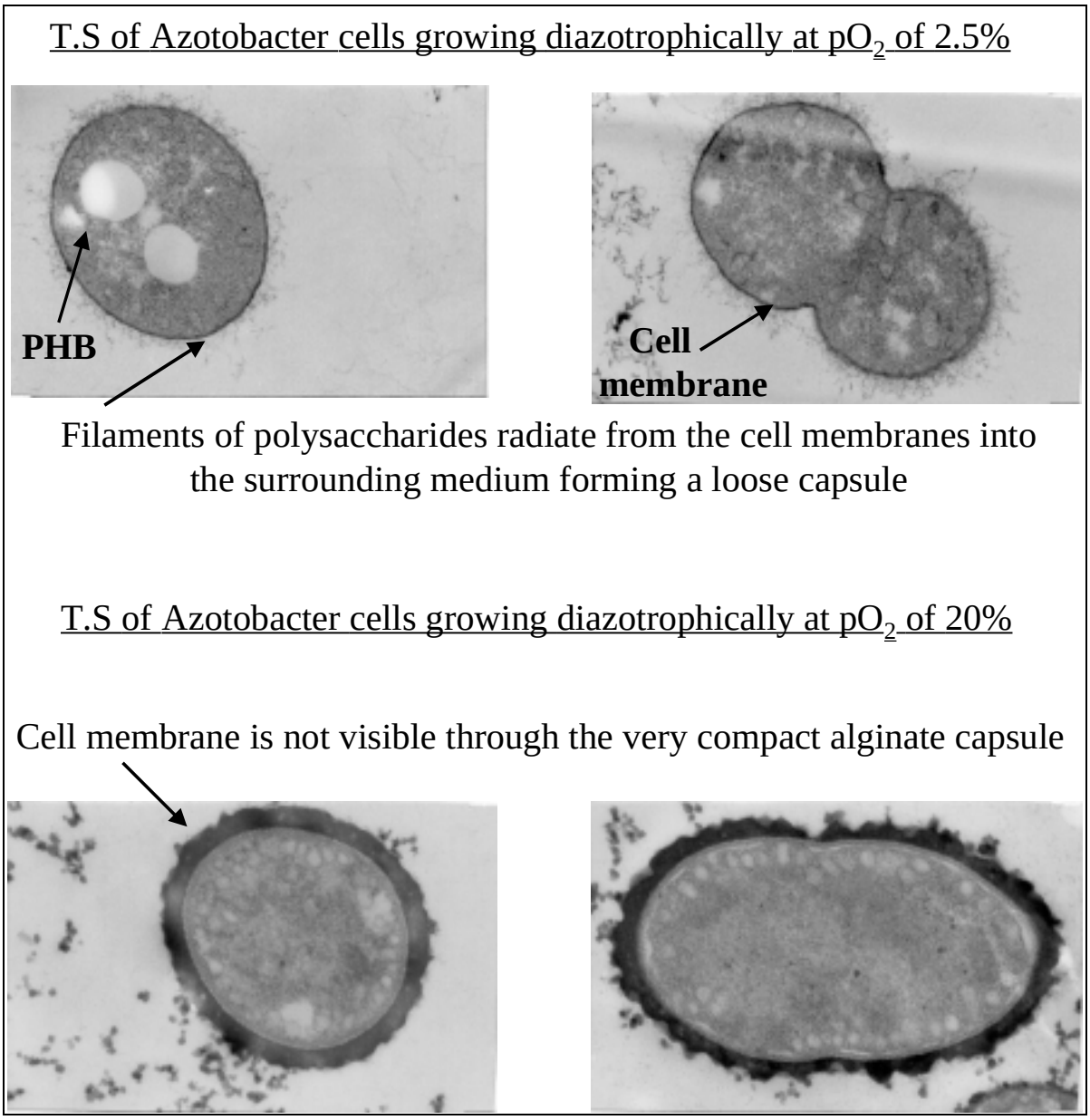


Fig. 4.48 Thin sections of *A. vinelandii* cells grown diazotrophically at pO2 value of 2.5 and 20% air saturation.

In fact, our experimental findings significantly add further weight to the latter argument. In addition, alginate production did not increase linearly with increasing pO_2 values, due to the fact that the dense alginate capsule around the cell exhibited not only a mass transfer barrier against oxygen but also to other media constituents hence lowering biomass and alginate productivity. Whereas, the softness and the diffusability of the alginate material around *Azotobacter* cells at an intermediate pO_2 value exhibited no mass transfer resistance to cultural nutrients and this permitted the continuous production of alginate.

It is therefore reasonable to claim that the alginate capsule structure may help to stabilize the intracellular oxygen concentration at a minimal value at which the nitrogenase system is not inhibited, i.e. the intracellular oxygen concentration in A. vinelandii is always stable at a specific range in expense to alginate composition and respiratory process.

5. CONCLUSIONS

In this study, growth characteristics of the nitrogen fixing bacterium *Azotobacter vinelandii* as well as some factors controlling the alginate production by this strain were quantitatively investigated. From a quantitative examination of growth parameters, respiration and morphological changes, relationships between the different essential processes, i.e. nitrogen fixation, respiration intensity, PHB biosynthesis and alginate formation, were established.

The first part of this study was focused on the production of alginate under controlled-microaerophilic conditions to examine the suitable oxygen concentration profile in respect to quantitative alginate formation for this strain. Testing a pO_2 range of 0-10% air saturation, showed that intermediate pO_2 values (2.5-5%) were optimal for alginate and biomass production. Above and below these values, the bacterium either wasted the carbon and energy source in respiration (CO2) or in PHB biosynthesis (44-58% of the biomass). Thus, efficient conversion of carbon source to alginate is achieved only if the DOT is accurately controlled at an intermediate value.

To lower the extreme O_2 sensitivity, different nitrogenous rich compounds were added to the mineral medium. Surprisingly, the cells exhibited a pronounced oxygen sensitivity even in the presence of complex nitrogenous materials. Furthermore, higher alginate yield ($Y_{alg/X}$) was obtained in nitrogen free medium, and for the purpose of medium simplicity the addition of nitrogenous compounds was not considered.

To gain more insights toward a better understanding of the inhibitory effect of oxygen towards nitrogenase and its relation to alginate biosynthesis, respiratory protection - one of the two mechanisms for nitrogenase protection - was impaired through phosphate limitation. This was performed in a pO_2 controlled bioreactor (2.5-3%) as well as in shaked flasks. The alginate production was sharply increased when the phosphate concentration was depleted from the medium, and 100mg/l (the lowest phosphate concentration used) was found to be the most suitable initial phosphate concentration for alginate production at a pO_2 of 2.5%. A productivity yield of 0.21g/l*h was obtained in low phosphate medium (100mg/l) compared to 0.17 and 0.14g/l*h at 200 and 400mg/l phosphate, respectively. In the phosphate limited phases, a relation was found between the RQ of the culture and the efficient conversion of

sucrose to alginate. Namely, a lower RQ value was accompanied with higher alginate productivity.
Since the control of the dissolved oxygen tension was found to be critical for the shift in metabolism, growth rate and alginate production, the physiology of this bacterium cannot be reproducibly studied in pO_2 – uncontrolled systems (flasks).

The theoretical RQ value for alginate as well as PHB biosynthesis were then predicted based on the biochemical reaction leading to their biosynthesis. Values of 0.8 and 1.33 should be the optimum RQ values for alginate and PHB production from sucrose, respectively.

To test the applicability of the former RQ calculations, a quantitative study was performed using *Azotobacter* in phosphate limited continuous culture with different DOT levels (1-10%). Highest specific alginate productions (at pO_2 of 2-5%) were accompanied by values of RQ around 0.8. Those RQ values were, indeed, near to the theoretical optimal RQ value for alginate production. The specific rate of sucrose consumption q_S increased in direct proportion with dilution rates (for each pO_2 value) and the sugar used for maintenance at different pO_2 values were extrapolated. It was found that the sugar used for maintenance (m_s) increased when pO_2 values of the culture were increased, and from the carbon balance, it was concluded that most of the maintenance sugar was directed to protect the cells form excess oxygen through respiration.
This was also shown from the specific oxygen uptake rate which increased with increasing DOT up to 5% and then surprisingly remained constant till 10% air saturation. This phenomenon could not be explained by respiratory protection, suggesting the involvement of another not yet identified factor.
Oxygen consumption rates for maintenance (m_o) were also increased with increasing the DOT and reached it's maximum of 15 mmol O_2/g biomass /h at pO_2 of 10%.

It was also found that higher DOT serves to induce the formation of good quality alginate having a higher molecular weight and a higher guluronic acid residue content.

The production of alginate was further optimised in a fed batch culture under sucrose limited condition. It was reasonable to conclude that, fed batch operation was favourable for better alginate quality but not for a better alginate productivity.

From the above results and based on the morphological observation of capsules around the cells even in the presence of high shear stress exerted by the agitation speed in the bioreactor, a new explanation for the biological role of alginate was proposed. In addition to the previously two reported mechanisms for nitrogenase protection (respiration and reversible inactivation of nitrogenase enzyme), acts alginate biosynthesis in diazotrophically growing culture of *A. vinelandii* also as a direct physical barrier exerting a mass transfer resistance of oxygen molecules into the cell.

For testing the validity of this hypothesis, capsule thickness as well as its structure was morphologically studied in phosphate limited continuous culture with different agitation speeds and with different DOT values. Surprisingly, capsule formation was also observed at the highest shear stress applied (1000rpm). However, agitation speed of 600 rpm was found to be the optimum for alginate production (in term of alginate yield -$Y_{alg/X}$ of 0.8g/g). The optimum alginate yield was parallel to the highest specific capsule formation, which indicates the existence of a hydrodynamic effect.

Since non-capsulated cells were not observed with increasing the shear stress, capsule formation was concluded to play an important role for the survival of the diazotrophically growing cells in phosphate limited and pO_2 controlled microaerophilic conditions.
The structure of the capsular polysaccharides, however, was dependant on the dissolved oxygen tension applied. The capsular material was more dense and compact at higher pO_2 value (20% air saturation) whereas loose and not compact at lower pO_2 value (2.5% air saturation). The pronounced effect of DOT on alginate epimerase explains these results.

In conclusion, the production of alginate by diazotrophically growing *Azotobacter vinelandii* was not only affected by the culture medium but also by the bioreactor hydrodynamics. Under conditions of oxygen stress (nitrogenase inactivation) the bacterium produced compact alginate capsules to stabilise the intracellular oxygen concentration at a minimal value suitable for nitrogenase activity. The presence of such a layer around the cell may also limit other cultural constituents to diffuse and hence lower biomass and decreased q_{alg} at higher DOT values were measured.

6. ZUSAMMENFASSUNG

Im Mittelpunkt dieser Arbeit steht die Untersuchung der Wachstumseigenschaften des stickstoffixierenden Bakterienstammes *Azotobacter vinelandii* sowie die Quantifizierung einiger die Alginatbiosynthese beeinflussender Faktoren. Quantitative Untersuchungen vor Wachstumsparameter, Atmung und morphologischen Veränderungen ermöglichen die Klärung des Zusammenhanges zwischen essentiellen Prozessen wie Stickstoffixierung, Atmungsaktivität, PHB-Biosynthese und Alginatproduktion.

Das für die quantitative Alginatproduktion optimale Sauerstoffkonzentrationsprofil wurde unter kontrollierten mikroaerophilen Bedingungen ermittelt. Untersuchungen zum Einfluß verschiedener Konzentration von gelöstem Sauerstoff (pO2: 1-10%) sowohl im Hinblick auf die Alginatausbeute als auch in Bezug auf die Biomasse ergaben einen Sauerstoffbereich von 2.5-5%. Ein geringer oder höherer pO_2 führte zum Verlust von Kohlenstoff zugunsten der PHB-Synthese (44-58 % der Biomasse) bzw. zur Veratmung zu CO_2. Die effiziente Umwandlung der Kohlenstoffquelle zu Alginat ist nur durch die genaue Kontrolle einer intermediären Gelöst-Sauerstoffkonzentration möglich.

Um die extreme Empfindlichkeit des Stammes gegenüber Sauerstoff zu senken, wurden dem Mineralsalz-Medium verschiedene stickstoffreiche Verbindungen zugegeben. Es konnte überraschenderweise gezeigt werden, daß die Zellen sogar in Gegenwart von Stickstoffverbindungen eine deutliche Sauerstoffempfindlichkeit aufwiesen. Außerdem wurde im stickstofffreien Medium eine höhere Alginatausbeute ($Y_{alg/x}$) erzielt, so daß zur Vereinfachung des Mediums auf die Zugabe von Stickstoffverbindungen verzichtet wurde.

Um die Erkenntnisse der inhibierenden Wirkung von Sauerstoff auf die Nitrogenase und deren Zusammenhang mit der Alginatbiosynthese zu vertiefen, wurde der ‚Atmungsschutz', einer der beiden Mechanismen zum Schutz der Nitrogenase, durch Phosphatlimitierung im pO_2 kontrollierten Bioreaktor (2,5-3%) sowie in Schüttelkolben beeinträchtigt. Die Phosphat-Erschöpfung im Medium erhöhte die Alginatproduktion erheblich und 100mg/l (die niedrigste eingesetzte Phosphatkonzentration) erwies sich als beste Startkonzentration für die Alginatsynthese bei einem pO_2-Wert von 2.5%. Eine gute Ausbeute von 0,2g/l*h wurde in

einem phosphatarmen Medium (100mg/l) erzielt, im Gegensatz zu 0.17 und 0.14 g/l*h bei 200 und 400mg/l Phosphat. Ein Zusammenhang zwischen dem Atmungquotienten (RQ) und der Effizienz des Sucrose-Umsatzes zu Alginat wurde in phosphatlimitierten Wachstumsphasen festgestellt. Die Gelöst-Sauerstoffkonzentration war eindeutig für diese Verlagerung des Stoffwechsels verantwortlich. Auf der Grundlage dieser gewonnenen Erkenntnis waren Untersuchungen im pO_2-unkontrollierten System (Schüttelkolben) irrelevant.

Basierend auf den biochemischen Reaktionsgleichungen, die zur Alginatsynthese führen, wurde der theoretische Atmungsquotient berechnet. Werte von 0,8 und 1,33 wurden demnach als optimale RQ Werte für die Alginat- bzw. PHB- Produktion aus Sucrose bestimmt.

Um diese theoretischen Werte experimentell zu verifizieren, wurden quantitative Untersuchungen mit *A. vinelandii* in einer phosphatlimitierten kontinuierlichen Kultur mit verschiedenen Gelöst-Sauerstoffkonzentration (1-10%) durchgeführt. Die maximale spezifische Alginatbildungsrate (pO_2=2.5%) zeigt Atmungquotient-Werte von ca. 0,8. Die spezifische Sucroseverbrauchsrate q_s nahm proportional mit der Verdünnungsrate (für jeden pO_2 Wert) zu. Wenn die pO_2 Werte der Kultivierung erhöht wurden, stiegen der für die Zellerhaltung metabolisierte Zucker (m_s) an. Die Kohlenstoffbilanzierung deutete darauf hin, daß dieser Zucker verbraucht wurde, um die Zellen gegen den Überschuß an Sauerstoff zu schützen. Bestätigt wurde diese Feststellung durch die spezifische Sauerstoffaufnahmerate, die mit zunehmender Gelöst-Sauerstoffkonzentration (bis zu 5%) anstieg und danach überraschenderweise bis zu 10% Luftsättigung konstant blieb. Dieses über Atmungschutz unerklärbare Phänomen deutete auf einen weiteren bislang nicht identifizierten Faktor hin.

Sauerstoffverbrauchsraten für die Zellerhaltung (m_0) nahmen ebenfalls proportional mit der gelösten Sauerstoffkonzentration zu mit einem Maximum von 15mmol O_2/g Biomasse/h bei einem pO_2-Wert von 10%. Höhere Gelöst-Sauerstoffkonzentrationen führten eindeutig zu einem qualitativ besseren Alginat mit einem höheren Molekulargewicht und einem erhöhten Guluronsäure-Restgehalt.

Im Weiteren wurde die Alginatproduktion in einer Fed-Kultivierung unter Sucrose-Limitierung optimiert. Das zeigte, daß die Fed-Kultivierung für eine bessere Alginatqualität jedoch nicht für eine erhöhe Alginatproduktivität geeignet war.

Ergänzend zu den bereits erwähnten Ergebnissen hat die morphologische Untersuchung des Stammes und die Beständigkeit der Schleimschicht um die Bakterienzelle trotz der hohen Scherkräfte im Bioreaktor – hervorgerufen durch die erhöhte Drehzahl – zu einer neuen Erklärung der biologischen Rolle von Alginat geführt. Zusätzlich zu den bereits bekannten Mechanismen des Nitrogenase-Schutzes (Atmung und die reversible Inaktivierung des Nitrogenase Enzyms) wirkt die Alginatproduktion in diazotroph wachsenden Kulturen von *Azotobacter vinelandii* als direkte physikalische Barriere, die einem Sauerstofftransport in die Zellen Widerstand leistet.

Um diese Theorie zu validieren, wurde die Dicke der Schleimschicht sowie deren Struktur morphologisch in einer phosphatlimitierten kontinuierlichen Kultur mit verschiedenen Drehzahlen und mit unterschiedlichen DOT werten untersucht.

Eine Schleimschichtbildung wurde überraschenderweise auch bei der maximal eingesetzten Drehzahl (1000rpm) beobachtet. Eine Drehzahl von 600rpm erwies sich als optimal für die Alginatproduktion (Alginatausbeute $Y_{alg/X}$ =0,8g/g). Die optimale Alginatausbeute verlief parallel zur höchsten spezifischen Schleimschichtbildung, was auf einen hydrodynamischen Effekt hindeutet. Da Zellen ohne Schleimschicht auch bei erhöhten Drehzahlen nicht beobachtet werden konnten, spielt diese Exopolysaccharidbildung offensichtlich eine wichtige Rolle für das Überleben dieses Stammes in diazotroph kultivierten Zellen unter Phosphatlimitierung und pO_2-kontrollierten microaerophilen Bedingungen. Die Struktur des Polysaccharides war hingegen abhängig von der eingesetzten gelösten Sauerstoff Konzentration. Die Schleimschicht war dichter und kompakter bei höheren pO_2-Werten (20% Luftsättigung) und locker bei pO_2-Werten von 2,5% Luftsättigung. Der ausgeprägte Einfluß von der gelöst Sauerstoffkonzentration auf die Alginat-Epimerase erklärt diese Ergebnisse.

Die Produktion von Alginat mit *Azotobacter vinelandii* wird also nicht nur von dem Kultivierungsmedium sondern auch von der Hydrodynamik im Bioreaktor beeinflußt. Unter Sauerstoffstress produziert das Bakterium kompakte Alginatschichten um die intrazelluläre Sauerstoffkonzentration bei einem minimalen Wert, der sich für die Nitrogenaseaktivität eignet, zu stabilisieren. Diese Schicht um die Zellen limitiert jedoch auch die Diffusion anderer Bestandteile des Mediums und führte dadurch zu einer Abname sowohl von Biomasse als auch q_{alg} bei höheren DOT Werten.

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8. APPENDIX

Tab. 8.1 Fermentation run with pO_2 of 10% air saturation.

time	A600	Biomass dry weight G/l	Viscosity K	Alginate g/l	Consumed sugar g/l	PHB g/l
0	0.232	0.50	0	0	0	0
3	0.818	0.70	0	0.2	11.66	0
6	2.724	1.25	2.04	0.31	11.78	0.1
9	9.029	3.38	3.87	1.04	19.6	0.12
12	15.188	5.30	12.21	1.98	37.9	0.3
15	13.135	4.70	12.49	1.09	38.3	0.2
18	12.255	4.45	9.31	1.2	39.2	0.15

Tab.8.2 Fermentation run with pO_2 of 5% air saturation.

Time h	A_{600}	Biomass dry weight g/l	Viscosity K	Alginate g/l	Consumed sugar g/l	PHB g/l
0	0.232	0.50	0	0	0	0
3	1.962	1.09	0	0.2	2.7	0
6	2.724	1.09	0	0.25	4.8	0.3
9	9.029	2.9	0.79	0.3	9.5	0.6
12	12.402	3.35	0.81	0.573	12.4	1.3
15	15.598	4.24	7.65	1.04	16	1.5
18	23.223	6.04	42.5	1.63	21.6	2.3
21	27.504	7.21	175	2.56	28.9	2.59
24	33.370	8.45	320	2.89	37.9	3.26
27	22.959	5.25	190	2.7	38.5	3

Tab. 8.3 Fermentation run with pO_2 of 2.5% air saturation.

Time h	A_{600}	Biomass dry weight g/l	Viscosity K	Alginate G/l	Consumed sugar g/l	PHB g/l
0	0.232	0.50	0	0.2	0	0
6	2.842	0.89	0	0.16	10.4	0.5
12	9.264	2.43	4.9	1.54	-	1.15
15	17.827	4.49	8.84	1.88	15.5	2.01
18	19.968	4.93	48.7	2.5	20.58	2.3
21	23.545	6.00	195.5	2.6	25.7	2.45
23	25.891	6.65	201	3	28.2	2.6
25	29.117	7.35	301.9	3.4	33.6	3
27	31.023	7.79	350	3.5	39.3	3.21

Tab. 8.4 Fermentation run with pO2 of 0-1% air saturation**.**

Time h	A_{600}	Biomass dry weight g/l	Viscosity K	Alginate g/l	Consumed sugar g/l	PHB g/l
0	0.32	0.53	0	0	0	0
6	0.03	0.43	0	0	7.3	0
12	1.38	0.69	0	0	10.5	0.2
15	2.70	0.94	0	0.2	10.6	0.4
18	4.69	1.13	3	0.3	10.9	0.9
21	6.42	1.21	3.02	0.32	11.2	1.4
24	6.68	1.20	3.8	0.3	11.8	1.5
27	8.15	1.70	2.8	0.4	13.2	1.5
30	12.26	2.30	2.1	0.6	14.6	2.3
33	14.31	2.49	2	0.65	15.3	2.8
36	14.63	2.51	2	0.71	17.2	2.9
48	21.46	3.24	2.03	0.69	23.3	4.5
54	24.87	3.90	3.89	0.8	25.9	5.0
60	28.53	4.95	12.12	0.78	31.6	5.2
72	33.96	5.68	9.31	0.67	39.1	6.3

Tab. 8.5 Fermentation run with 400mg/l phosphate concentration.

Time h	OD_{600}	Residual PO_4^{3+} mg/l	Biomass dry weight g/l	Alginate g/l	Consumed suagr g/l	PHB g/l
0	0.15	400	0.43	0	0	0
7,3	0.25	342	0.33542	0.2	7.82	0.11258
10,3	2.4	324	0.61232	0.27	9.5	0.59768
13	5.89	272,6	1.6185	0.45	14	0.7215
16,28	14.25	175	4.3195	0.9	23.8	0.7605
18.2	24.65	88.3	7.657	1.85	25.2	0.783
20.47	27.98	27	9.40215	2.18	30.2	0.79785
23.2	36.89	20	12.3875	3	36.1	0.8325
25	45.25	10.3	14.6131	3.53	0	0.8469

Tab. 8.6 Fermentation run with 200mg/l phosphate concentration.

Time h	OD_{600}	Residual PO_4^{3+} mg/	Biomass dry Weight g/l	Alginate g/l	Consumed sugar g/l	PHB g/l
0	0.32	200	0.224	0,2	0	0
3	0.23	191.8	0.276	0,3	5	0
6	0.6	168.1	0.224	0,48	7.5	0,2
9	1.21	104	0.602	0,36	13	0.23
11	3.75	14	1.479	0,7	18	0.195
13	9.2	2.37	3.265	1.14	23	0.26
15	16.98	0	5.923	1.78	27.8	0.36
17	22.98	0	7.65	2.5	32.1	0.45
19	28.98	0	9.43	2.98	35.8	0.42
21	27.36	0	8.94	3.18	38	0.46
23	28.8	0	9.705	4.06	40	0.52

Tab. 8.7 Fermentation run with 100mg/l phosphate concentration

Time h	OD_{600}	Residual PO_4^{3+} mg/l	Biomass dry Weight g/l	Alginate g/l	Consumed sugar g/l	PHB g/l
0	0.3	100	0.184	0,2	0	0
2	0.26	100	0.26	0,23	5	0
4	0.45	100	0.316	0,23	4.2	0
6	0.61	92	0.628	0,2	7.7	0
8	0.48	85	0.584	--	9.55	0
10	1.68	68	0.96	--	15.4	0
12	2.1	46.5	0.97	1	17.5	0.15
14	5.6	25.4	2.078	1,2	20.5	0.23
16	9.15	1	3.5	1,4	24.5	0.2
18	12.98	0	4.67	2,36	30.5	0.21
20	16.1	0	5.59	3,4	35.5	0.19
22	18.35	0	6.43	4.4	38	0.195
24	22	0	7.42	4.98	39.5	0.18

Tab. 8.8 Phosphate limited continuous culture with different dissolved oxygen tensions and dilution rates**.**

D h^{-1}	PO_2 %	Biomass dry weight g/l	Alginate g/l	PHB g/l	Consumed. Sugar g/l	**RQ** -	**QO2 QCO2 (mmol/l*h)**	
0.08	1	1.32	0.65	0.56	6	1.18	7.45	8.80
	2.5	1.57	0.91	0.5	12.6	0.87	26.43	23.00
	5	1.45	0.97	0	15.28	0.832	42.06	35.00
	10	0.98	0.43	0	15	1.27	31.57	40.10
0.15	1	1.09	0.59	0.01	3.69	1.2	9.33	11.20
	2.5	1.56	1.12	0.2	8.79	0.82	36.58	30.00
	5	1	0.99	0	8.37	0.811	39.45	32.00
	10	0.9	0.52	0	9	1.15	37.39	43.00
0.22	1	0.8	0.2	0	2.75	1.09	11.82	12.89
	2.5	1.04	0.975	0	5.67	0.9	33.33	30.00
	5	0.98	0.958	0	7.33	0.83	51.80	43.00
	10	0.54	0.43	0	4.78	1.09	32.11	35.00
0.26	1	0.98	0.32	0	2.91	1.12	13.39	15.00
	2.5	0.8	0.6	0	3.82	0.98	26.53	26.00
	5	0.7	0.61	0	5.11	0.815	46.6	38.00
	10	0.45	0.2	0	3.53	0.84	29.7	25.00

Tab. 8.9 Continuous culture with different agitation speeds

Agitation Speed Rpm	Biomass dry weight g/l	Alginate g/l	PHB g/l	Consumed sugar g/l	QO2 QCO2 Mmol/l*h		RQ _
300	0.7	0.27	0.02	3.80	9.73	15.66	1.61
400	0.78	0.45	0.01	4.00	14.85	15.89	1.07
500	0.89	0.6	0.0086	5.00	19.89	17.71	0.89
600	1.09	0.89	0.006	6.50	28.73	22.70	0.79
800	1	0.56	0.0069	6.11	37.27	24.97	0.67
1000	0.9	0.29	0.0054	5.80	75.67	27.24	0.36